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Original Article

Preparation of Melatonin Nanoparticles and their Enhancement of Lungs Bioavailability in the Treatment of Lungs Cancer

Niyaz Ahmad[1,2,*], Mohammed Saifuddin Khalid[3], Ayman Makki Al Ramadhan[1], Mohammed Riyad Al Hammad[1], Mohd Faiyaz Khan[4], Ziyad Saeed Almalki[4], Faisal K Alkholifi[5], Zabih Ullah[6], Mohd Ibrahim Khan[7], Nazia Khan[8]

[1]Department of Pharmaceutics, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam, SAUDI ARABIA.
[2]Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, INDIA.
[3]Department of Pharmacology, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam, SAUDI ARABIA.
[4]Department of Clinical Pharmacy, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al kharj, SAUDI ARABIA.
[5]Department of Pharmacology, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al Kharj, SAUDI ARABIA.
[6]Department of Pharmaceutical Sciences, College of Pharmacy and Dentistry, Buraydah Colleges, Alqassim, SAUDI ARABIA.
[7]Department of Pharmaceutical Sciences, SIHAS, Sam Higginbottom University of Agriculture Technology and Sciences (SHUATS), Allahabad, Uttar Pradesh, INDIA.
[8]Department of Pharmaceutical Sciences, Ibn Sina National College for Medical Studies, Jeddah, SAUDI ARABIA.

ABSTRACT

Background: To develop a first-time-novel polymeric NPs from PLGA and their coating by Chitosan (CS) of Melatonin (MT) for the enhancement of lungs bioavailability by targeting lungs from the nose. **Materials and Methods:** The Melatonin LC-MS/MS method was developed for the first time for a comparative Pulmokinetic evaluation administered by different routes which will be useful for the treatment of lung cancer and *in vitro* evaluation on lung cancer cell-lines (H1299). The solvent evaporation method was used to develop the PLGA nanoparticles and their encapsulation by CS. **Results:** CS MT PLGA MPs were given a significantly high release and permeation of MT as compared to MT PLGA NPs. Different routes of administration were used to compare the pulmokinetics parameters. Lung cancer cell lines (H1299) were used for anticancer activities. PDI, ZP, PS, DL, and EE of CS MT PLGA NPs were observed at 0.131±0.009, +19.5±1.37mV, 98.36±7.15 nm, 4.19±0.38%, and 74.16 ± 5.29% respectively. Mucoadhesive nature of CS MT PLGA NPs was showed greater than MT PLGA NPs and MT S with 0.303 retention time with mas' spectra of 233.20/174.10. The developed method was linear (1–1000ng/mL) with inter and intra-day accuracy (91.68–98.95%) followed by precision (1.67—2.69%). C_{max} and AUC_{0-24} were significantly enhanced (p<0.001) than i.v. and oral in the lungs. **Conclusion:** Capping of CS on PLGA NPs could be a strong possible nanocarrier for Melatonin to enhance their solubility of a drug (MT), entrapment, sustain and controlled release, and stability, with their therapeutic application.

Keywords: Melatonin, CS MLT PLGA NPs, Mucosal Delivery, Lung cancer, LC-MS/MS, Comparative pulmokinetics for lungs.

Correspondence:
Dr. Niyaz Ahmad
Department of Pharmaceutics, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam 31441, SAUDI ARABIA.
Email id: niyazpharma@gmail.com

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INTRODUCTION

Lung cancer is the foremost reason of related-cancer mortality and morbidity in the world peoples.[1] International Agency for Research on Cancer reported on 2018; that people died every year 1.7 million from lung cancer; which just doubles the death report compared to other cancers.[2] Lung cancer is diagnosed in 80.0% of cases on the basis of Non-small-cell lung cancers (NSCLC) reports.[3] There was a surgical stripe that can be due to the adjunction of chemotherapy from platinum which is standard care. It also offered the patients the best care for a long time due to the surgical procedure for diseases who can endure the surgery.[4] There is a day-by-day advancement in the description of NSCLC that has also been directed to targeted immunotherapies. It also has the objective to prolong the survival of those with metastatic or locally advanced lung cancer.[5] In spite of optimal treatment, though, reappearance occurs in so high a proportion of patients.[6]

A natural health substance (Melatonin) was used in the treatment of many types of cancers. It has been established anticancer activity in many laboratories based on observational studies, and also in several randomized clinical- trials.[7-9] Mills and their team assessed relative risks of mortality in a 2005 systematic review

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and meta-analysis based on results of clinical trials (n=9) which studied multiple solid tumors and observed a 34.0% reduction of relative risk.[9] Melatonin has given a very strong effect when it was used with the combination of chemotherapy.[10-11] There were also some effects reported for antioxidant of Melatonin in the clinical trials.[12-13] One review article reported recently updated one more effect of Melatonin with the combination of chemotherapy significantly decreased the frequency &/or severity of leucopenia, asthenia, vomiting, nausea-, thrombocytopenia-, and hypotension.[14] This is a requirement of confirmation which also validated its strong evidence of clinical research. But nowadays only one way to solve this problem and care for most NSCLC patients will perform surgical resection.[4] In this type of population; it's a great requirement for demanding clinical research. Most of the people are using generally Melatonin and it was suggested >50.0% of the time postoperatively for people having lung cancer through naturopathic doctors and they are focusing on this type of cancer care.[15]

There is a great attraction of researchers nowadays; they used nose–to–lungs for targeting purposes and to improve the bioavailability of lungs to escape the hepatic first-pass metabolism by using the non-invasive route followed by the easy administration of drugs.[16-19] Finally, we can say that PLGA NPs will be an alternative and best method to deliver the drugs to the lungs via an intranasal route. Poly (lactic-co-glycolic acid) (PLGA) is approved by the US-FDA that is biocompatible and biodegradable and is a copolymer of polylactic and polyglycolic acid. PLGA was used for the so many preparations of macromolecules, small molecules of drugs, RNA, DNA, and peptides for drug delivery systems in a controlled way. Polymeric degradation mainly depends on the molecular weight of the polymer with the ratio of lactide and glycolide followed by relevant physicochemical properties.[20] There are so many types of PLGA and PLGA (50:50) is biodegraded quickly.[21]

The negative charge presents on PLGA NPs surface potentially which degrades their mucoadhesive property and reduces the drug's bioavailability. The burst release of drugs is unavailable the drugs at targeted the site which is the most important disadvantage of PLGA-based NPs that showed very limited therapeutic action. The researchers applied their efforts for the modification of PLGA NPs surface with the help of CS to prepare the coating of PLGA NPs from CS for the increment of mucoadhesion and cellular uptake due to their positive charge surface.[22-25] These surface-modified NPs give us a useful way of applications as other pharmaceutical preparations delivered via nose–to–lungs. Drugs are protected due to the encapsulation of PLGA first after that CS that means drugs are not directly involved in the environment of biological degradation for the enhancement of drugs-bioavailability into the lungs.

The most important point related to the evaluation of the lung's pulmokinetics parameters of MT (i.e. AUC, C_{max}, K_{el}, T_{max}, and Half-life, etc), is the lack of availability of suitable LC-MS bioanalytical method in the lungs but all the methods reported on analysis of food or medicinal or herb plants except one i.e. human saliva method.[26] One more important point is don't forget to quantify the MLT in the lungs, therefore the requirement of a bioanalytical method is very necessary to find out the MLT quantity up to the nanogram. The researchers have developed a new LC-MS method, validated and applied it successfully for the Pulmokinetics parameters analysis based on US-FDA guidelines. The validated method has given so many benefits like a very small retention time with the highest sensitivity including the highest efficiency with a very small total run time.

Researchers have developed for the first time a capped novel CS MT PLGA NPs to target Melatonin for the lungs from nasal drug delivery and enhance the bioavailability of MT in the lungs followed by enhancing their therapeutic level. We have also increased the MT-therapeutic level by using the optimal dose to avoid systemic exposure to Melatonin. We have also found the highest solubility and permeability with optimized CS MT PLGA NPs as compared to MT PLGA NPs. The characterization of CS MT PLGA NPs was performed by various physico-chemical parameters for the evaluation of targeted MT delivery to lungs from nasal permeation. We have also delivered CS MT PLGA NPs from various routes of delivery like intranasal, oral, and intravenous to establish a comparative evaluation of pulmokinetics in Wistar rats. LC-MS technique was used to develop a bioanalytical method to determine the concentration of Melatonin in the Lungs in the lungs-cancer-treatment with cancer cell lines. The cell lines [non-small cell lung carcinoma (NSCLC: H1299)] were used to evaluate the performance of newly optimized CS MT PLGA NPs based on cytotoxicity activity.

MATERIALS AND METHODS

We have purchased Melatonin from AK Scientific, USA; PLGA and chitosan from Tokyo Chemical Industry Co. Ltd., Japan. Most of the chemicals like DCM and other analytical solvents (Methanol, Ethanol, Acetonitrile, etc) are from Sigma-Aldrich, USA. Milli-Q-Water was used for all types of preparations and analysis.

Nanoparticles Development

We have developed the PLGA NPs which were loaded with Melatonin by the use of the solvent evaporation method.[27,28] We have dissolved the 40 mg Melatonin in the already prepared 3.3% PLGA polymeric solution which was in DCM (5.0 mL) so as to become an organic phase. We prepared the PVA (10 mL, 0.5%, w/v) as an aqueous phase in a separate preparation. The emulsification of the organic phase was prepared by the addition of an aqueous phase with the help of a syringe (0.3 mL/min) by the ultrasonic processor (Fisher Scientific Technology, USA) for 3.0 min at voltage-efficiency (60.0% W). The magnetic stirrer

was used to evaporate the organic solution for six hours. We have collected MT PLGA NPs after high-speed centrifugation and freeze-dried (Lab Conco., LPYH, Lock 6, USA freeze dryer) followed by 3.0-times washing by the use of deionized water. We have added 2.0% chitosan solution prepared in acetic solution (0.50%) in a particular quantity of MT PLGA-NPs for 2.0 hr at room temperature. We have also washed 2-times after the high-speed centrifugation for Capped CS MT PLGA NPs and then redispersed in the Milli-Q-water. Finally, Coated CS MT PLGA NPs were dispersed and then stabilized by the use of Lab Conco Lyophilizer, the USA for three days at a temperature of −60.0°C. Final nanoformulation was prepared in triplicate i.e. CS MT PLGA NPs.

Optimized CS MT PLGA NPs Characterization

Poly Dispersity Index, ZP, and particle size

Measurement of PS is a very important parameter to evaluate the prepared nanoformulation. Therefore, PS enhances then reduces the surface area of PS to the drug -absorption. PDI, ZP, and PS were measured by Malvern–Zetasizer, the UK by techniques depending on the light scattering for the prepared nanoformulations. Before going to measure the PS of NPs, everyone should do the dilution. We should maintain temperatures of 25°C and 90°C fixed the scattering angle before going to analyze the PS. Therefore, we have diluted all the samples before analysis of PS and their ZP of prepared NPs in the triplicate measurement of all prepared formulations. All the samples were analyzed in triplicate for their PS and ZP measurements.[29]

Scanning electron microscopy (SEM)

We performed the studies for the determination of the shape and size of NPs by the use of SEM purchased from FEI, INSPECT S50, Czech Republic.[29]

%EE: Entrapment Efficiency, LC (%): Loading Capacity, and Process Yield (%)

LC and EE of NPs were determined by the ultracentrifugation for 30.0 min at 15,000 rpm at 4.0°C. We have determined the results related to EE (%) and LC (%) of nanoparticles by the ultracentrifugation for thirty minutes at 15,000.00 rpm at 4°C. We have determined the Melatonin free-quantity from the supernatant

$$EE\ (\%) = \frac{\text{Whole MT Quantity} - \text{Free MT Amount}}{\text{Whole MT Quantity}} \times 100$$

$$LC\ (\%) = \frac{\text{Whole MT Quantity} - \text{MT Free Amount}}{\text{NPs Weight}} \times 100$$

by the use of the LC-MS method (our in-house-developed method). We have used below mentioned equation for the calculation of EE (%) and LC (%) for developed-novel-NPs:[29]

As per the below-mentioned formula; we calculated Process Yield (%):

Entrapment of Melatonin study by DSC

DSC 214 Polyma, Germany was used to examine the samples of MT (Pure Melatonin), MT PLGANPs, and CS MT PLGA NPs. Each and every sample weighed first 10mg and put inside the DSC-aluminium-pan and now crimped. We have analyzed all the samples in-between the range of temperatures from 20-400°C. We have started the heating 10°K min^{-1} rate with the help of supplying the Nitrogen-gas continuously.[29]

A released study of drug (Melatonin)

MT released (*in vitro*) a study was performed with slight modification by 2-different mediums (pH: 7.4 & 5.5) to introduce the physiologic and acidic-pH environment of cancer cells and it was released from MT PLGA NPs and CS MT PLGA NPs.[29] We have placed the same amount (25mL) of MT PLGA NPs in a separate dialysis bag in 2-different mediums (pH: 7.4 & 5.5). We have also started the same-release evaluation of MT in a similar set of CS MT PLGA NP samples. All the analysis was performed from a paddle-type dissolution apparatus at 37°C with a speed of 100rpm. We collected every aliquot (1mL) at a predetermined sampling time point which was exchanged by the same volume of fresh buffer. Every sample was withdrawn 1mL at every time point. All the withdrawn samples were filtered via a 0.2µm syringe filter. These samples were analyzed by LC-MS/MS method (in-house developed).

A nasal mucosa permeation (ex vivo) study

The nasal tissues were collected from the goat's nasal cavities freshly from the slaughterhouse. Melatonin was permeated in a fixed 0.785 cm^2 area for tissue cells (Logan Instrument Corporation, Piscataway, NJ). A phosphate buffer (20mL) was taken (7.4pH at 37°C) into the receptor chamber. A 10mg Melatonin of MT PLGA NPs and CS MT PLGA NPs were taken separately in the donor chamber after twenty minutes as pre-incubation time for every analysis. The samples were taken 500.0 µL from receptor the chamber on the basis of already decided time intervals. All the withdrawn samples were filtered via a 0.2µm syringe filter. These samples were analyzed by LC-MS/MS method (in-house developed).[29]

Animal study

Ethical approval (IRB-UGS-2021-05-125) has taken from IRB of Imam Abdulrahman Bin Faisal University (IAU) committee for the analysis of PK evaluations. We have taken albino rats containing weight 200 to 250 gram maintained the natural light

$$\text{Process Yield (\%)} = \frac{W_1 \text{ (Dried NPs Weight)}}{W_2 \text{ (overall weight of dried initial material)}} \times 100$$

and dark cycle. All the rats are easily reach their food and water at 20-30.0°C temperature maintained with humidity 50-55.0%. 200—250g wt Albino rats were collected and kept in the cages based on predetermined groups. We kept all the rats in their cages as encoded-groups. We started our research study at the time of wake up when rats are freely moving.

A bioanalytical MDMV based on UHPLC-MS/MS

We strictly adopted the US–FDA guidelines, 2001 for MDMV of MT. We have taken a total of eight points for the standard or calibration curve to plot the linearity curve for the peak area ratio calculation and $1/x^2$ regression was used. We used the signals to noise (10:1) to calculate the LLOQ and LOD. The authors have taken out from the deep freezer six already spiked for the extraction of samples. After the extraction of samples, we plot the graph between mean area response versus post-extraction spiked MT-free in the matrix (lungs). The authors have examined the accuracy and precision (inter and intraday) by choosing all QCs (LLOQQC, LQC, MQC, and HQC: six replicates for each) and one calibration curve in lungs homogenate samples.

Conditions for UPLC–MS/MS

We used highly sensitive and great resolution Mass i.e. LCMS-8050, ESI, Triple Quadrupole, Kyoto, Japan with Cyano C_{18} (Pinnacle DB, 1.9 µm; 30 × 2.1 mm) column for the quantification of MT in which UHPLC was binary-solvent-manager with autosampler. Cyano C_{18} (Pinnacle DB, 1.9 µm; 30 × 2.1 mm) column was used with methanol: formic-acid (0.10%):: 98:02, 0.300ml/min, injection-volume (2.0µl), and 1.0 min total run time. We have applied the mass detector that contains a highly-sensitive and maximum-resolution tuneable mass-detector i.e., LC-MS-8050, ESI-Triple-Quadrupole, Kyoto, Japan. We applied the mass conditions like scan-time: 1-min, inter-scan delay: 0.02 sec with 0.10µ scan step: 30,000 µ/s, and collision gas was argon gas. MT was quantified with –13.5 eV collision energy and +ve ion mode. The mass transition of MT was shown in Figures 1A and 1B. Lab Solution Software Version 5.93 (Kyoto, Japan) was used to examine the amount of Melatonin. CC and QC were prepared freshly with unknown samples of homogenate of lungs were formulated and taken for analysis (Figure 1C, 1D).

Preparation of calibration curve with quality control samples

We prepared the Melatonin stock solution first (1mg/mL). We have made the eight standards dilutions [in the mixture of water: methanol:: 1:1] from the main MT stock solution and then 2% spiking in the 98% of blank lungs homogenate to prepare the spiked CC and QC. We have decided the calibration range i.e., 1.0 to 1000.0 ng ml^{-1}. We have followed the same method of spiking procedure for all QCs like [HQC (850), MQC (450), LQC (2.85), and LLOQC (1.02)]. We stored all freshly spiked samples in the refrigerator at a temperature (2-8°C).

Lungs homogenate samples extraction

All the fresh CC, QCs, and unknown lungs homogenate samples have been taken collectively for pulmokinetics analysis. We have taken 630.0µl each aliquot in the fresh dried cleaned glass tube. We added 2% formic acid (250.0µL) and then vortexed the aliquots at 300 rpm for six minutes for the break-down of matrix protein. After that the breaking of the matrix protein, we added an extraction mixture (3.5mL dichloromethane: DCM+1.5mL ethyl acetate) to the solution. We have centrifuged all the samples

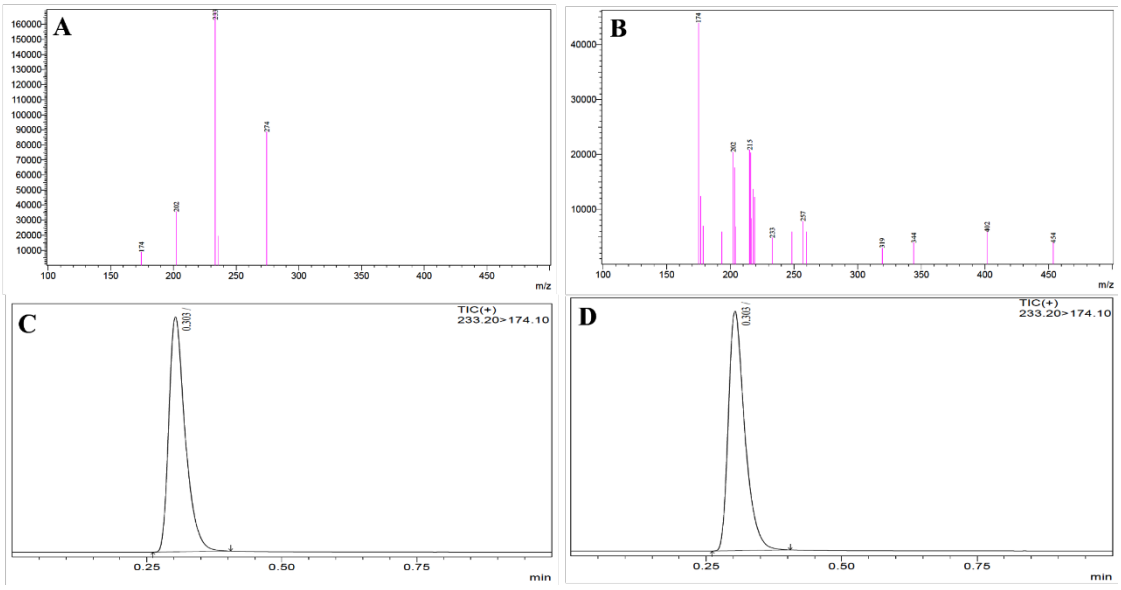

Figure 1: MS Scan [A], MSMS Scan [B], chromatograms of extracted lung-homogenate [C] and plasma extracted [D] of Melatonin.

at 4500 rpm for ten minutes at 4.0°C. After the centrifugation, we transferred the 3.0 mL supernatant into another fresh glass tube. After that, all the supernatant samples were dried under a nitrogen stream on a water bath containing a temperature 37±1°C. All the sample residues have been reconstituted with a 650 µL mobile phase with the help of vortexing the samples at 350 rpm for five minutes. We transferred the reconstituted residues into a fresh and clean HPLC (1.5 mL) vial for pulmokinetics analysis.

Pulmokinetics

A comparative pharmacokinetics was studied for the CS MT PLGA NPs by using various routes of administration (-i.v., oral-, and -intranasal) in Wistar rats. We will compare and determine the best route for Melatonin loaded CS PLGA NPs in the targeting of lung drug delivery. We have taken three groups (6×3=18) for the evaluations of pulmokinetics parameters to determine the Melatonin quantity by the delivery of CS MT PLGA NPs. We have decided to carry out the pulmokinetics study based on sampling points -0.25, 0.5-, 1.0, -2, 4-, -8, 12- and 24.0 hr. Total eighty-one animals were used i.e., 27 × 3 = 81 which were taken and divided as G-1: i.v., G-2: oral, and G-3: intranasal. We have sacrificed the three rats at each sampling-time point to dissect the lungs to make the lungs homogenate for LC-MS-based pulmokinetics analysis.

Cytotoxicity Study

Investigational agents are generally used to determine cytotoxicity based on the prediction of a researcher on the various cancerous cells. They used so many techniques like bioassay (*in vitro*) first which gives us a basic idea of the screening of any predicted agents. It has also given us their potential effects.[30] The technique (*in vitro* bioassay) was the first time predicted to perform cytotoxicity by using an investigational agent (e.g.MT) in various cells that will provide an opportunity for screening and safety of an evaluation of the investigational agent (e.g.MT).[30] Thus, we conducted the MTT colorimetric evaluation on the NSCLC cell line (H1299) for free drug (MT), CS MT PLGA NPs, and placebo CS PLGA NPs. H1299 cells i.e. 6×10^3 particular number of cells prepared for all the well i.e. 96-well plate twenty-four hours' time-duration. We applied different concentrations i.e., 5 to 100.0 µg/ml: free-MT, CS MT PLGA NPs, and CS PLGA NPs (No Drug) for the incubation of the cells. After the treatment of twenty-four hours of incubating samples, we removed the cells from the wells. We incubated the cells with the concentration of 0.50%, w/v MTT solution (25.0 µL) for up to four hours at 37°C. MTT solution which is not reacted was kept off after the incubation and then DMSO (150 µL) is used to cells were to expose the cells in every well for twenty minutes duration to dissolve the formed formazan crystals. Formazan solution showed their absorbance at 570nm by the use of a microplate reader (Thermo Fisher Scientific, Waltham, MA) which was proportionate to the number of viable cells in the wells. The treated cells showed viability on the basis of calculation and their comparison with control cells and reported.

Statistics Application

The results will be calculated by the value of Mean±SEM (Standard-Error of Mean). Student's *t*-test was applied to evaluate any variation by the use of ANOVA-technique based on *p*–value.

RESULTS

Characterizations of optimized Nanoparticles

We prepared the PLGA NPs by the use of PLGA and PVA via the method (i.e., emulsification solvent evaporation) in which PVA was taken as a stabilizer. In this research, the ultrasonication technique was used for the reduction of particle size to nanometer size with smaller PDI. On basis of the results, PLGA NPs given the range of particle size i.e., 76.82±3.69nm but after the loading of the drug (MT) didn't show any very high enhancement of the size of loaded MT PLGA NPs i.e., 77.39±5.48 nm. On the other side, we coated the MT PLGA NPs from chitosan that showed a significant increment in the size of particles i.e., 98.36±7.15 nm (Table 1). But for the PDI results for PLGA NPs (No drug) are just opposite to the PS, they showed the reduction of PDI i.e.,

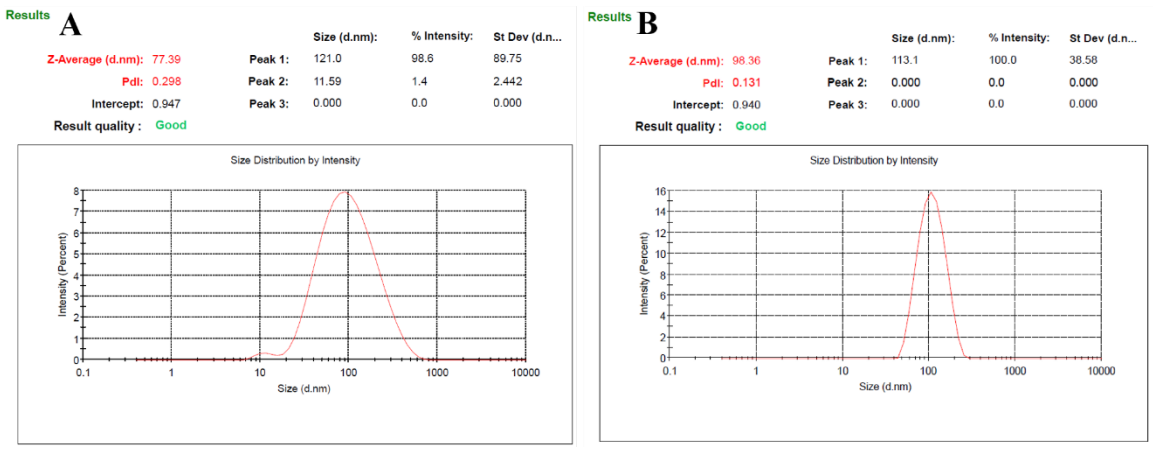

Figure 2: Particle size examined through diffraction light scattering (DLS) technique [A]: Melatonin PLGA NPs and [B]: CS Melatonin PLGA NPs.

Table 1: Optimization of Nanoparticles with their characterizations.

Nano formulation	Drug Amount used	PLGA (%)	PVA (%)	Chitosan (%)	Particle Size (nm)±SD	PDI	ZP (mV)	DL (%) ± SD	EE (%) ± SD
Placebo PLGA NPs	-	3.3%	0.5%	-	76.82±3.69	0.303±0.003	–12.0±1.83	-	-
MT PLGA NPs	40 mg	3.3%	0.5%	-	77.39±5.48	0.298±0.012	–14.1±1.76	3.98 ± 0.21	68.43 ± 4.97
CS MT PLGA NPs	40 mg	3.3%	0.5%	2.0%	98.36±7.15	0.131±0.009	+19.5±1.37	4.19 ± 0.38	74.16 ± 5.29

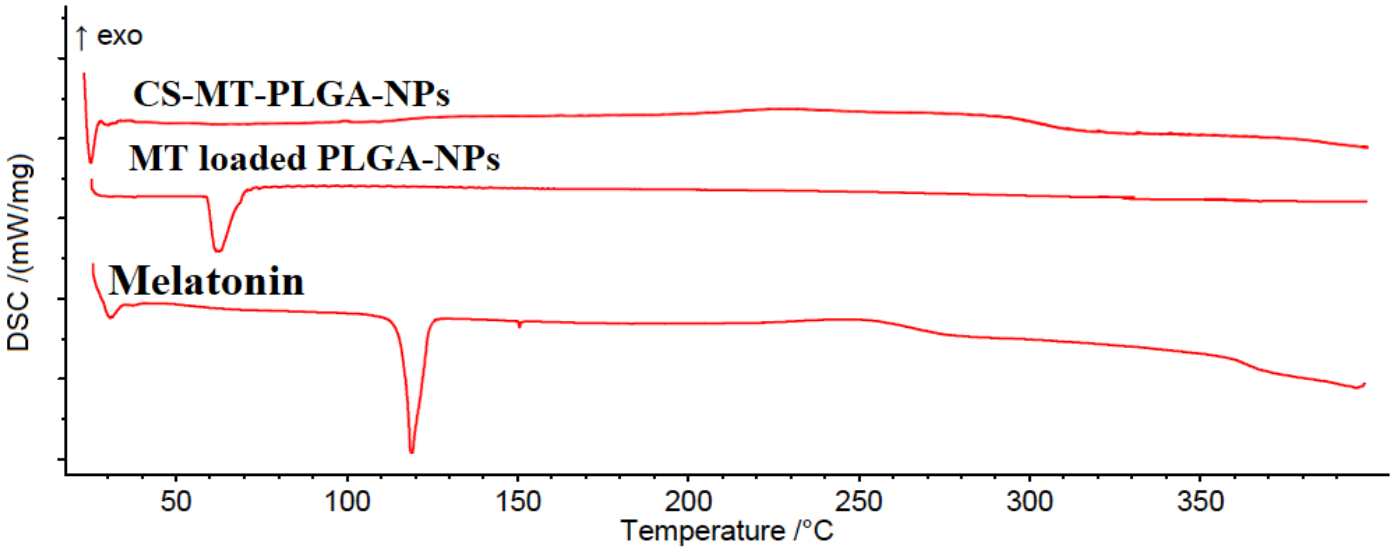

Figure 3: A comparative DSC thermograms for pure Melatonin and their optimized nanoformulations.

0.131±0.009 for capping of CS on MT PLGA NPs and also for loading of drug into the PLGA NPs i.e., 0.298±0.012. Finally, we got the results in all the cases, PDI was less than 0.303±0.003 which clearly showed the monodisperse nature of the particles. We found the results related to the surface charge of PLGA NPs and MT PLGA NPs (–12.0±1.83 and –14.1±1.76) were negative as shown in Figure 2 and Table 1. We have also found a significant change in the surface charge of NPs after the coating with CS i.e., negative PLGA NPs and MT PLGA NPs (–12.0±1.83 and –14.1±1.76) to positive +19.5±1.37 mV (for CS MT PLGA NPs) and also increases the stability of the NPs (Figure 8).

Differential scanning calorimetry (DSC)

Figure 3 showed free Melatonin and dried NPs from lyophilizer (MT PLGA NPs and CS MT PLGA NPs) of DSC thermograms. Pure Melatonin has given a sharp endothermic transition Peak at 118.6°C which co-relates with its melting point. MT PLGA NPs didn't show any Melatonin peak. This is clear confirmation to us; Melatonin is completely entrapped inside the core of MT PLGA NPs. But there is a very typical peak shown in-between 45.0°C to 55.0°C in the pure polymer of PLGA and also MT PLGA NPs (Figure 3). There were no peaks were observed in opt CS MT PLGA NPs. We didn't find any DSC thermograms of MT and PLGA DSC in the CS MT PLGA NPs that can be the coating of chitosan over PLGA NPs. Therefore, we concluded that the Melatonin was completely encapsulated inside the opt NPs (Figure 3).[34]

A released study for Melatonin (*in vitro*)

A drug is released from drug-loaded prepared nanoformulation like NPs to produce the intended physiological response. We can modify their physiological response by the modification of the physicochemical medium. We have studied Melatonin delivery from the CS MT PLGA which is enclosed-MT inside the cancer microenvironment. We performed our released evaluation on two different pH: 5.5 and 7.4 which can be resembled the tumor microenvironment pH, different kinds of cellular compartments, and also to the nasal mucosa. The release profile of Melatonin from nanoformulations is shown in Figure 4 on two different pH. We have found that Melatonin release was 66.84±6.01% and 81.38±7.01 % for the PLGA NPs and CS-PLGA NPs respectively at 7.4pH. Capped CS MT PLGA NPs have given 44.64±3.33% Melatonin burst release as compared to MT PLGA NPs was 36.41±2.97% at four hours. Melatonin was found to be more released initially in CS MT PLGA NPs as compared to MT PLGA NPs with a sustained and controlled release at 7.4pH. Melatonin

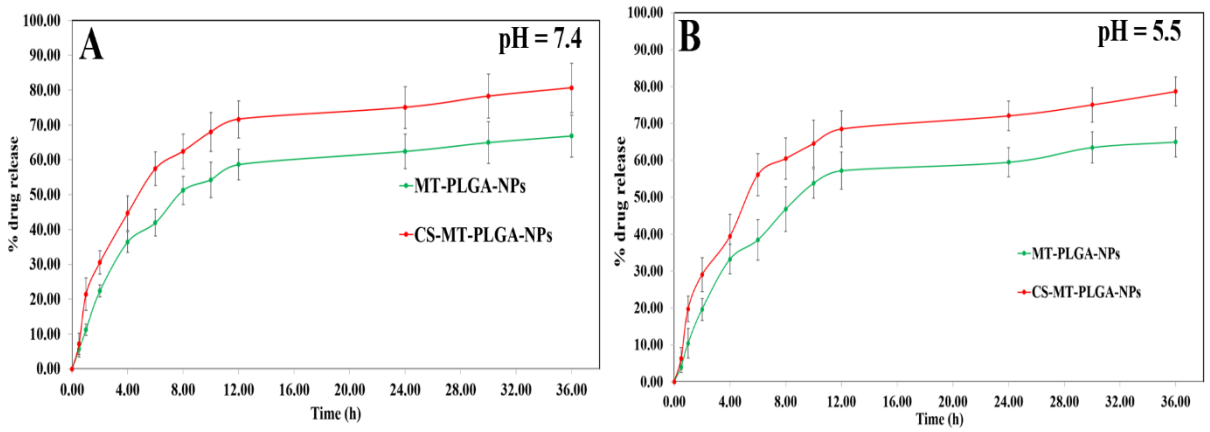

Figure 4: A comparative %release of Melatonin (MT) on different pH from optimized NPs.

was released very fast from CS MT PLGA NPs (39.41±5.99%) as compared to MT PLGA NPs (33.21±4.01%) at 5.5pH. On the basis of these results, CS MT PLGA NPs showed clearly that have pH-responsive properties. We have found that 78.66±3.97% MT released from CS MT PLGA NPs a sustained and controlled release upto 36 hr at 5.5pH as shown in Figure 4B.

Nasal Permeation Evaluation

CS MT PLGA NPs have given very highly significant results for permeation i.e. $p<0.01$ when compared to MT PLGA NPs. CS MT PLGA NPs have shown greater permeation (83.64±5.87%)) than MT PLGA NPs (65.78±6.34%) (Figure 5). The positive charge presents on the surface of chitosan due to the presence of an amino group at the C2 position which has given a great interaction with the cell membranes having a negative charge on it. So, in this way, MT exhibited increased nasal permeation. On the basis of these observations, the epithelial mucosal cells have also taken a part to facilitate the mechanism of their tight junctions.[33] CS MT PLGA NPs showed less than 100nm particle size with their lipophilic surface property that can be simply permeated to the biological membrane. They have already reported drugs with lipophilic properties permeated highly in the nasal mucosa or skin.[42] Finally, we have reached out to give our opinion smaller size of NPs with maximum % yield, highest EE (%), and highest DL (%) is principally responsible for the highest-permeation of CS MT PLGA NPs which will give suggestions to the greater production for animal study.

LC-MS based Bioanalysis

Mass spectra and chromatograms of Melatonin were shown in Figure 1. Plasma and lungs homogenate extracted chromatograms has displayed in Figures 1C and 1D. MT extracted recovery results were more than 82.64% for LH and plasma. We plotted the linearity graph in the lungs homogenate which showed the R^2 > 0.989 in the range of 1-1000ng/ml. LOD and LOQ were found 0.863 and 1 ng/ml. All the chromatograms for lungs homogenate

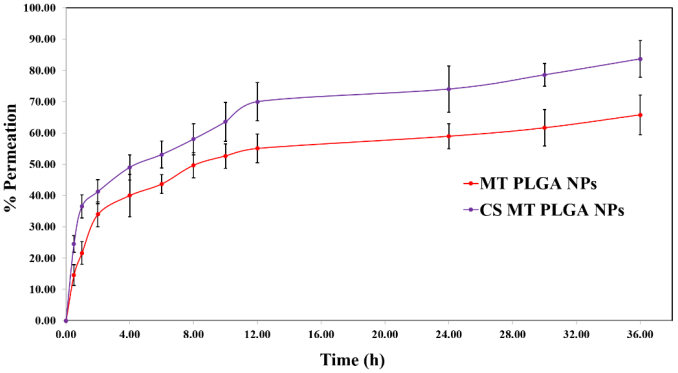

Figure 5: *Ex vivo* permeation profiles of opt CS MT PLGA NPs as compared MT PLGA NPs via goat nasal mucosa.

of Melatonin were optimized based on the selectivity results. All the lungs homogenate QCs for inter and intraday showed their results were found to be %CV (1.20–2.49% and 1.31–2.69%) and % accuracy (91.68–98.95% and 92.32–98.60%) in the in Table 2. All the lung's homogenate QCs showed their all stability results in Table 3 which were within the acceptance limits.[34,43]

Pulmokinetics Results Examination

This is a first-time determination of pulmokinetics results for Melatonin from the delivery of CS MT PLGA NPs that was given from various routes i.e., i.v., oral, and intranasal, and also established their comparison. We identify how much of Melatonin quantity reached into the lungs as shown in Figure 6 and Table 4. T_{max} was observed at 2 hr in which given a maximum concentration of Melatonin i.e., C_{max}: 619.97±44.9ng/g for intranasal with highly significant results$^{###}$ and then intravenous showed significant results$^{##}$ for C_{max}: 475.34±33.9 ng/g but orally showed lowest C_{max} results i.e., 299.67± 32.40 ng/g. We have found the best results from i.n. for Melatonin quantity in the lungs (Figure 6 and Table 4). In this way, it was proved that CS MT PLGA NPs enhanced the bioavailability of Melatonin in the lungs from the intranasal route as compared to MT-S. We have got the results that proved for the delivery of Melatonin via intranasal from CS MT PLGA NPs still needs to establish and perform the clinical study.[16-19]

Table 2: Melatonin Data for Precision and Accuracy.

	Intra-batch				Inter-batch		
QC ID	Theoretical content (ng/ml or ng/g)	Mean concentration Observed (ng/ml or ng/g)	Accuracy[a] (%)	CV[b] (%)	Mean concentration Observed (ng/ml or ng/g)	Accuracy[a] (%)	CV[b] (%)
LOQQC	1.02	0.98±0.012	96.08	1.22	0.99±0.013	97.06	1.31
LQC	2.85	2.82±0.061	98.95	2.16	2.81±0.064	98.60	2.28
MQC	450.00	431.34±5.19	95.85	1.20	439.19±7.32	97.58	1.67
HQC	850.00	779.24±19.37	91.68	2.49	784.94±21.09	92.32	2.69

Values (Mean ± SD) are derived from 6 replicates: [a]Accuracy (%) = Mean value of [(mean observed concentration)/(theoretical concentration)] × 100; [b]Precision (%): Coefficient of variance (percentage) = standard deviation divided by mean concentration found×100; Theoretical contents; LOQQC: 1.02 ng mL^{-1}, LQC: 2.85 ng mL^{-1}; MQC: 450 ng mL^{-1}; and HQC: 850 ng mL^{-1}.

Table 3: Stability Parameters for Validation Data of Melatonin in Lungs Homogenate.

Exposure condition	LQC (2.85ng/ml or ng/g)	MQC (450ng/ml or ng/g)	HQC (850ng/ml or ng/g)
Long term stability; recovery (ng) after storage (–80°C)			
Previous day	2.84±0.04	438.09±19.64	841.64±23.19
30th day	2.78±0.05 (97.89%)	411.39±14.08 (93.91%)	815.68±19.67 (96.92%)
Freeze–thaw stress; recovery (ng) after freeze–thaw cycles (–80°C to 25°C)			
Pre-Cycle	2.83±0.03	440.45±18.17	838.74±21.01
First Cycle	2.76±0.04 (97.53%)	415.27±15.48 (94.28%)	821.64±20.67 (97.96%)
Second Cycle	2.71±0.05 (95.76%)	401.89±9.34 (91.25%)	813.67±22.07 (97.01%)
Third Cycle	2.67±0.03 (94.35%)	392.52±11.27 (89.12%)	799.46±21.42 (95.32%)
Bench top stability; recovery (ng) at room temperature (25°C)			
0.0 hr	2.82±0.05	442.37±20.64	836.13±16.47
24.0 hr	2.77±0.06 (98.23%)	409.05±11.38 (92.47%)	816.52±13.79 (97.65%)
Post processing stability; recovery (ng) after storage in auto sampler (4°C)			
0.0 hr	2.84±0.06	432.84±13.67	837.41±18.61
4.0 hr	2.75±0.05 (96.83%)	420.19±14.13 (97.08%)	827.98±12.44 (98.87%)

Table 4: Pulmokinetic of Melatonin for CS MT PLGA NPs as compared with different routes of administration after the administration of single dose (10.0 mg kg^{-1} b. wt.) (mean ± SD; *n* = 6).

Parameters	CS MT PLGA NPs (i.v.)	CS MT PLGA NPs (oral)	CS MT PLGA NPs (i.n.)
C_{max} (ng/ml or ng/g)	475.34±33.97$^{\#\#}$	299.67± 32.40	619.97±44.93$^{\#\#\#}$
T_{max} (h)	2.00	2.00	2.00
AUC_{0-t} (min ng/ml) or min ng/g)	4912.65±169.19$^{\#\#}$	2689.01±171.43	10096.71±177.31$^{\#\#\#}$
$t_{1/2}$ (h)	11.53	11.92	38.04
K_e (h^{-1})	0.06011±0.00007	0.05813±0.00006	0.01822±0.00003
$AUC_{0-\infty}$ (min ng/ml) or min ng/g)	6553.16±301.34$^{\#\#}$	7843.94±166.76	27337.06±198.37$^{\#\#\#}$

AUC_{0-t}: Area under lung's concentration to time curve; C_{max}: maximum lungs concentration at time t; $t_{1/2}$: half-life; and K_e: Elimination Rate Constant. Data are represented as mean±SD (*n* = 6). Significantly high AUC and C_{max} were attained with CS MT PLGA NPs (i.n.) $^{\#\#\#}$(p<0.001), followed by CS MT PLGA NPs (i.v.) $^{\#\#}$(p < 0.01).

Table 5: Cell viability of H1299 cancer cells after 24 hr exposure of MT-S, CS MT PLGA NPs, and CS PLGA NPs (blank/placebo/No drug) nanoparticles.

MT concentration (µg/ml)	5.0	25.0	50.0	75.0	100.0
CS PLGA NPs (No Drug)	99.67±2.98	98.15±2.68	98.09±3.11	97.89±3.69	97.68±2.71
CS MT PLGA NPs	74.98±5.06	63.16±6.74	50.09±5.69	38.94±5.32	29.94±4.28
MT S	82.07±7.99	72.64±6.37	60.25±5.81	59.87±5.09	45.89±3.89

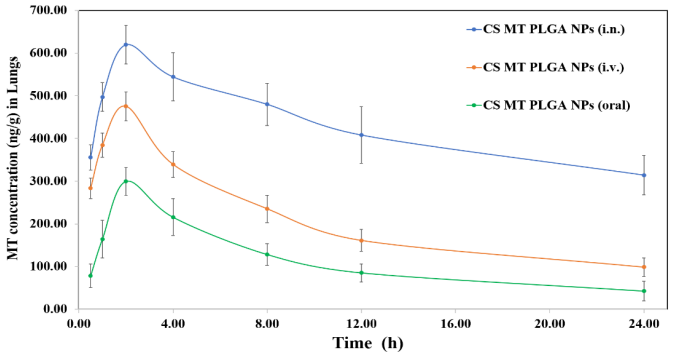

Figure 6: Pharmacokinetic study of Melatonin in lungs for CS MT PLGA NPs as compared with different routes of administration.

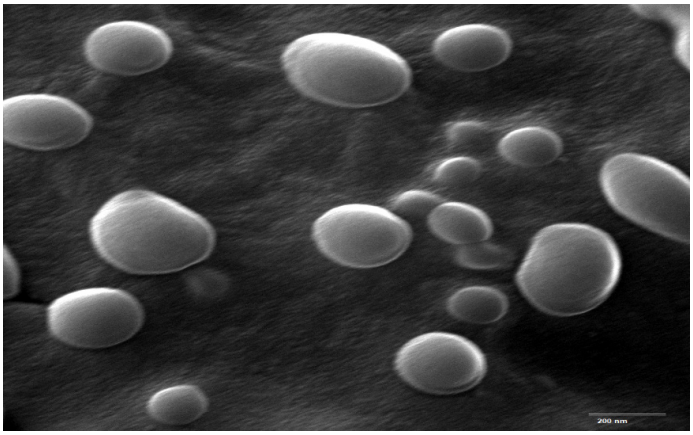

Figure 7: Scanning electron microscopy (SEM) images of CS MT PLGA NPs.

Establishment of MT and CS MT PLGA NPs for Cytotoxicity Study

There is the most important view for the cytotoxic agents that how many cells death occur during the performing the cancer cell line study in the discovery of cancer. Therefore, we selected Melatonin as a cytotoxic agent to determine its cytotoxic potential with optimized CS MT PLGA NPs on the NSCLC cell line (H1299).[44] We used an MTT assay agent i.e., 3-(4, 5- dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide to determine the cytotoxicity. We have tested Melatonin solution in a free form and CS MT PLGA NPs on the human non-small cell lung cancer cell line H1299 to examine the cytotoxicity of both agents that depending on how many cells die and their proliferation. We have identified the results after the twenty-four hours of MT S and CS MT PLGA NPs post-treatment which gives us a response depending upon their concentration. These results were evaluated based on the cell viability or toxicity against H1299 cells (Table 5). We got a smaller number of cell deaths in lower concentrations as compared to higher concentrations (50-100µg/ml) for the MT S and CS MT PLGA NPs. In the same way, we studied all the effects (cytotoxic or cell death) for CS MT PLGA NPs showed highly significant results ($p \leq 0.05$) when compared with MT-S (Table 5). CS MT PLGA NPs showed the best results for the Melatonin cytotoxic potential compared with MT-S. We got a higher value of IC_{50} for MT-S (96.89 µg/mL) than CS MT PLGA NPs (64.36 µg/mL). On the basis of observation and calculations, we got the CS MT PLGA NPs showed a maximum cytotoxic effect than MT-S. We didn't find any type of cytotoxic effects with Placebo nanoformulation (CS PLGA NPs) (Table 5).

DISCUSSION

The surface charge of CS MT PLGA NPs was plus (+) charge due to the presence of amino group on the chitosan which proves the MT PLGA NPs were coated with chitosan. This positive charge of CS MT PLGA NPs on their surface increases the adhesive property and retention on the cancer cells because the surface of cancer cell membranes contains the negative charge.[31] The phagocytes reduced the interactions of CS-coated NPs and also their absorption by these cells because for this reason least absorption of positively charged NPs.[32] Figure 7 showed smooth surface and spherical CS MT PLGA NPs by the scanning electron microscopes results. Entrapment Efficiency was estimated in percentage for MT PLGA NPs with the help of the equation mentioned in the Methods section. MT %EE was found to be a different proportion because of a change in % concentration of PLGA. On the other side, CS MT PLGA NPs were optimized and showed 74.16 ± 5.29% EE and 4.19 ± 0.38%DL. Capping of MT PLGA NPs from CS increases the mucoadhesive and staying time in the nasal mucosa. Another most important aim of this research is to determine the *in vitro* MT release-, permeation (*ex vivo*)-, pulmokinetics study-, and cytotoxicity study on H1299 cells cell lines. On the basis of our results and calculations, chitosan was coated on MT PLGA NPs for the Melatonin-delivery by the intranasal route. CS MT PLGA NPs exhibited the +19.5±1.37 mV ZP in the Figure 8A, B. CS MT PLGA NPs showed a positive charge on their surface to facilitate and improve the mucoadhesion property plus improvement of residence time and reducing the mucociliary clearance of CS MT PLGA NPs from the nasal mucosa.[33,34] The polymeric PLGA was taken to formulate the MT PLGA NPs and PVA works as a stabilizer. PLGA NPs were formulated generally in the presence of PVA used as a stabilizer which can easily bind to polymeric PLGA due to PLGA interpenetration and PVA molecules.[34] Here, copolymer PVA contains the vinyl part and shows the lipophilic property via both

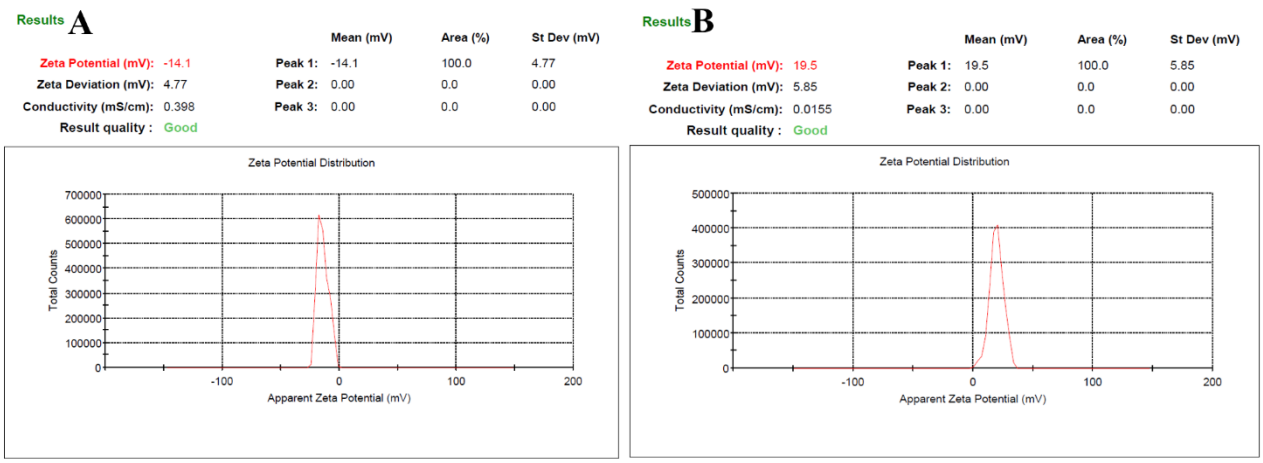

Figure 8: Zeta potential of [A]: Melatonin PLGA NPs and [B]: CS Melatonin PLGA NPs.

polyvinyl alcohol and polyvinyl acetate via blocking distinctively to the copolymer. PVA contains the lipophilic property which works as a penetrating agent for the organic phase when we were preparing the NPs. We evaporate the organic solvent from the matrix at the time of polymeric preparation which gives results, PVA entrapped inside the matrix of PLGA.[35] The particle size was reduced by the application of the ultrasonication technique in the dispersed phase. Here, NPs used as a nanocarrier having uniformly distributed in the nanoformulation within the nano-range of size i.e., 77.39±5.48. It was reported before the criteria of selection which are as follows: selection of polymer, copolymer, and selected of the drug at the time of preparation of NPs and the loading of the drug inside the PLGA NPs followed by the requirement of nano-range of size.[34] PDI showed very small values for CS MT PLGA NPs and their difference was also very small which means monodispersed all particles. Even at the time of loading of the drug (MT), there was a decrease in PDI (0.298±0.012), and all the formulations did not exceed the 0.303±0.003 PDI which represents the unimodal particle preparations.[36] PLGA-NPs and MT PLGA NPs were coated with CS a significant enhancement in particle size ($p < 0.05$). This will not increase in the PDI value as compared to uncapped NPs. At last, we concluded that there was no agglomeration in-between the particles after the capping of CS.

In addition to, this is the most important point to discuss surface charge and size of the nanoformulation is for the correlation of improved permeability and retention (EPR) effect. On the other side, the particle size is facilitated to prolong the retention time in the systemic circulation escaping the reticuloendothelial cells. Another side, the particle-charge is facilitating the targeted at a specific place and releases the drug.[37] Hence, the nanometric size of the proposed NPs preparation will give a passage to prolong systemic circulation that will support for passive targeting of the encapsulated Melatonin into the porous tumor area.[38] Another most important point to translocating the tumor cells from their surface due to the presence of negatively charged residue of phosphatidylcholine[39] which also facilitates the binding of positively charged particles. MT PLGA NPs surface charge was slightly positive in nature due to the presence of ionized 3-amide group and 5-alkoxy group are also the main ones responsible for the amphiphilicity of this molecule. PVA showed amphiphilic nature to form a stable fence on the surface of the polymer. This PVA fence shields the NP's surface charge and transfers the shear plane outer to the particle surface. Consequently, NPs' surface charge was verified as a slight negative zeta potential.[35] Additionally, chitosan has a weak base polysaccharide, consisting of β (1,4) linked monomers of N-acetyl-D-glucosamine and D-glucosamine, in which the surface amino groups are ionized in an acidic environment to provide a positive charge.[40] The chitosan has a positively charged amino group which in their structure has produced the positive surface of opt CS MT PLGA NPs. The change of negative surface charge of MT PLGA NPs has been shifted to the positive surface charge of CS MT PLGA NPs which is clearly shown in Figure 8. CS MT PLGA NPs showed a positive charge of the surface to facilitate the binding of the CS MT PLGA NPs to the negatively charged surface of the tumor cells at the time of prolonged circulation of the NPs inside the circulation due to the EPR effect.

If we modified the release of the enclosed drug inside the NPs is very beneficial for escaping the unfavorable side effects because of normal cells exposes at the time the release of drug. In this research, we proposed the optimized CS MT PLGA NPs were modified for the release of entrapped Melatonin in the cancer microenvironment. Therefore, for the achievement of our main objective, we have modified the surface of MT PLGA NPs by the coating of chitosan that has released the MT on a specific pH in the cancerous cells' atmosphere. This already established the correlation of the cancer microenvironment acidic pH i.e., 5.5-7.0 because of the hypoxic condition during uncontrolled proliferation, lacking of perfusion of blood, and glycolytic cancer cell metabolism.[41] Hence, it is a very important point for discussion related to the release of drugs in the acidic

environment and reduced their systemic exposure. Therefore, we studied the release of drug in two separate mediums i.e. 5.5 and 7.4 pH that nearly resembles the cancer environment and systemic circulation. CS MT PLGA NPs clearly indicated the fast release of MT in the acidic environment that are opposite of physiological pH i.e., slow. So, we can say and apply for the CS MT PLGA NPs can be stay more in the systemic circulation without the entrapped drug release. On the other site, CS MT PLGA NPs will reach into the site of action that the drug will release very quickly to apply for its physiological role.

CONCLUSION

Capped CS Melatonin PLGA NPs were developed through the solvent emulsion evaporation method. It was optimized by smaller PDI with very small particle size and spherical shape of NPs with maximum DL and EE. A sustain-release of Melatonin was successfully optimized. It was very helpful for the delivery of Melatonin from intranasal-to-lungs having no hepatic-first pass-metabolism. The optimized CS MT PLGA NPs showed higher up to 36 hr in the targeting of drug to lungs. CS MT PLGA NPs have a positive-zeta potential which was very helpful for the lung cancer cells to employ the increased cytotoxic potential of optimized preparation as compared to free-MT, CS MT PLGA NPs have exhibited a higher cytotoxic effect in the cell lines (H1299). Our in-house newly-developed bioanalytical (LC-MS) method was successfully applied to determine the pulmokinetics parameters for the different routes of delivery of CS MT PLGA NPs. The lung's homogenates extraction recovery of Melatonin was found to be more than 82.64%. All the results were found within the acceptable- range- of limits for all stability parameters of validation. CS MT PLGA NPs were successfully optimized which can be efficiently applied as a novel, non-invasive, effective, and safe treatment of lung cancer.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PLGA: poly(lactic-co-glycolic acid); **CS:** Chitosan; Melatonin: MT; **LC-MS/MS:** Liquid chromatography-mass spectrometry/mass spectrometry; **PDI:** Polydispersity Index; **ZP:** Zeta Potential; **PS:** Particle Size; **DL:** Drug Loading; **EE:** Entrapment Efficiency; **NSCLC:** Non-small-cell lung cancers; **US-FDA:** United States- Food and Drug Administration; **AUC:** Area Under Curve; **C_{max}:** Peak Plasma Concentration, **T_{max}:** Time to reach C_{max}; **NPs:** Nanoparticles; **SEM:** Scanning Electron Microscopy; **TEM:** Transmission Electron Microscopy; **K_{el}:** Elimination rate constant; **$t_{½}$:** Half-life; **LOD:** lower limit of detection; **LOQ:** lower limit of quantitation.

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