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EFFECT OF CHITOSAN COATING ON THE PHYSIOCHEMICAL CHARACTERISTICS OF GEFITINIB LOADED NANOLIPOSOMES

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Keywords:

Nanoliposomes, Zeta potential, Chitosan Coating, *in-vitro* release, Stability, Cytotoxicity study

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ABSTRACT: In the present work the nanoliposomes prepared by using REV and MEU methods were coated with different concentration of chitosan solutions. The main objective behind this work was to assess the physical properties, stability and in vitro release of uncoated and chitosan coated nanoliposomes of gefitinib. The existence of a covering on the exterior of nanoliposomes was confirmed by the modification in particle size and zeta potential. The chitosan coated nanoliposomes had shown significantly slow *in-vitro* drug release than uncoated nanoliposomes that confirmed the increased stability of nanoliposomes. The transmission electron microscopy, particle size analysis, FTIR studies, DSC analysis, and zeta potential studies were used to investigate the characteristics of uncoated and chitosan-coated nanoliposomes to develop and further optimize nanoliposomes that are directed for their systemic pharmacological applications. Stability studies and cytotoxicity studies of uncoated and chitosan coated nanoliposomes were performed. Chitosan coated formulations were found to be more stable and more cytotoxic than uncoated formulations. The ideal temperature was found to be 4 ºC.

INTRODUCTION: Nanoliposomes have ability to approach merely the precise cells, which is a prime requisite to accomplish preferred drug concentration at the target spot so that the undesirable effects can be minimized and optimum therapeutic effectiveness of drug on healthy cells and tissues can be achieved. They can also to protect the active moiety in blood circulation and deliver it at the targeted site at a sustained pace [1].

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Thus, nanoliposomes as a carrier helped in improving the therapeutic index of drugs by selective and controlled drug delivery, by declining the exposure of lethal drugs to susceptible tissue, and by controlling the drug pharmacokinetics and biodistribution.

All categories of drugs like hydrophobic, amphipathic and hydrophilic drugs are suitably delivered by using nanoliposomes as a carrier as it carried both lipophilic and hydrophilic environment in one system [2]. Moreover, nanoliposomes have found imminent applications in the various streams of nanotechnology like gene delivery, cosmetics, agriculture, food technology, diagnosis and cancer therapy.

High production cost, oxidation and hydrolysis of phospholipids, seepage and blending of encapsulated drug / molecules, less stability, small half-life, and squat solubility are some of the precincts of nanoliposomes. However, the instability of nanoliposomes *in-vitro* and *in-vivo* confines their application. The nanoliposomes have tendency to amassed and degrade, which causes seepage of encapsulated material during storage and washout rapidly through the system after intravenous injection. Literature suggested that, amid various factors that influence the stability of nanoliposomes, carrier's surface characters like fluidity, lipophilicity, and charge are of great significance. Therefore, the minute alteration in carrier's surface with polymers having required properties, we can easily improve the *in-vitro* and *in-vivo* steadiness of nanoliposomes.

Veneer with polymers of desired properties is an assuring approach of altering the surface characteristics of nanoliposomes, in which the nanoliposomes suspension was mixed simply with a polymer solution. Polymer coating improved the long-range mutual revulsion between contiguous bilayers and thus, enhanced the stability of nanoliposomes during storage. Various natural polymers like polysaccharides and synthetic polymers like polyvinyl alcohol, polyethylene and polyacrylamide have been used to amend the surface character of nanoliposomes which ultimately also improve the stability of nano-liposomes. Amid which chitosan is a positively charged polysaccharide and can be used to increase and modify the surface characters of nanoformulations, is also found to have a promising future in the medical and pharmaceutical fields.

Chitosan comprised mainly of glucosamine units and due to existence of amino groups it acts like a polycationic polymer. It is N-deacetylated derivative of chitin with anti-inflammatory and antioxidant properties [3, 4]. Tissue engineering, obesity control and drug development are its several important applications. During new drug formulation it used most widely as being biodegradable and biocompatible it also provide a protective capsule like safeguard to drug molecule [5]. Its chemical configuration and various suitable features like abundance, hydrophobicity, anti-microbial activity, low toxicity, biocompatibility, and biodegradability made chitosan an important ingredient to be used in the preparation various modified formulation and carriers like microsphere, microfilme, nano-particles, films, gels. As a carrier to entrapped and release active ingredient, it found applications in various fields like cosmetics, pharmaceuticals, food and biotechnology.

Literature suggested that various authors had used chitosan or allied polymers as a coating material for nano-formulations for targeting purposes and for improving their stability towards release of active moiety [6]. We recognized that suitable combination of the polymer based and lipid-based systems could amalgamate the advantages and diminish the disadvantages of each system, and thus lead to development of new system carrying reward of both systems [7].

In the current work, the nanoliposomes were prepared by using reverse-phase evaporation (REV) method using box-benkhen design and modified emulsification and ultrasonication (MEU) method using central composite design and then, the optimum batches from both methods were coated with diverse percentages of chitosan solutions. Then, the outcomes of diverse percentages of chitosan solution on zeta potential, particle size, and *in-vitro* drug release rate were studied. The transmission electron microscopy, FTIR studies, DSC analysis, particle size and zeta potential studies were used to investigate presence of chitosan coating on nanoliposomes. The characteristics of uncoated and chitosan-coated nanoliposomes were studied to develop and further optimize nanoliposomes that are directed for their systemic pharmacological purposes.

MATERIAL AND METHODS:

Materials: Soya lecthin was bought from CDH Chemicals, India. Gefitinib was gifted by Mac Chem Products India Pvt., Ltd., (Mumbai, India). Chitosan (molecular weight of 150 kD) was purchased from Fluka Chemika and was used as received. Analytical grade dimethyl sulphoxide, chloroform, disodium hydrogen phosphate, diethyl ether, tween 80 were obtained from Loba Chemie, India. Cholestrol was procured from Sigma aldrich. Ammonium acetate and acetonitrile (HPLC grade) were acquired from Fisher Scientific, Mumbai and

Rankem, India respectively. Every chemical used in preparation of formulation was of investigative grade. Deionization of water was done before use.

Cytotoxicity Study:

Cell Culture and Treatment: Cell lines studies were performed on human lung carcinoma cells (A549 and H1299) obtained from the National cell repository located at NCCS, Pune and was grown in DMEM media along with 10% fetal bovine serum (FBS) and antibiotics solution (IX Penstrip, Invitrogen). The cells were incubated at 37 ºC with 5% CO_2 and 95% humidity conditions.

The assay was specified by Mosmann in 1983 and is also known as cell viability assay. This in vitro colorimetric assay is based on cleavage of tetrazolium rings of pale yellow MTT by mitochondrial dehydrogenase enzyme from viable cells resulting in formation of dark blue formazon crystals. The formazon crystals get accumulated in healthy cells as they are impermeable to cell membranes. So these are solubilised using DMSO. The number of surviving cells is directly proportional to level of formazon product formed. The results of assay are read using Shimadzu double-beam spectrophotometer [8, 9].

MTT Assay: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed using 96-well plates; each well was filled by 100 µL media to which cell were treated with the synthetic compounds for 48 h. Lung cancer cells (A549 and H1299) were counted on the automated cell counter. About 8,000-10,000 cells were seeded in each well of the 96 well plates. The plate was incubated at 37 ºC with 5% CO_2 for 24 h. At the end of the 24 h, treatment was given to the cells in 3 concentrations of 1 µM, 5 µM, and 25 µM. The cells were further incubated for 48 h.

The media was removed from each well and MTT solution (5 mg / 10 mL) was added. This was incubated in the dark for 4 h. At the end of 4 h, the MTT solution was disposed from each well and the intracellular precipitate was dissolved in DMSO solution and the absorbance of the violet color formed as consequence of DMSO addition is read spectrometrically at 570 nm [8, 10]. The results were then represented as mean ± S.D obtained from three independent experiments.

Preparation of Chitosan-Coated Nanoliposomes: Nanoliposomes were prepared by reverse-phase evaporation (REV) method and modified emulsification and ultrasonication (MEU) method. In reverse-phase evaporation method, soya lecthin and cholestrol were dissolved in diethyl ether and gefitinib was dissolved in distilled water. The mixing of organic phase and aqueous phase was done in ratio (3:1, v/v), and a lipid film was prepared under reduced pressure at 40 ºC, using a rotary evaporator. Then 10 ml phosphate buffer solution (0.10 M, pH 7.0, PBS) containing tween 80 was added under a stream of nitrogen. Nanoliposomes were obtained by reducing the size of nanoliposomes using ultrasonication with a probe sonicator in an ice bath with 1s ON, 1s OFF intervals, for a total period of 10 min [11].

In modified emulsification and ultrasonication method, gefitinib ethanolic solution of a desired concentration was prepared by using anhydrous ethanol. The ethanolic solution of gefitinib containing lipid phase was heated on a water bath at 60 ºC. 10 ml of phosphate buffer (pH 6.8) containing tween 80 was maintained at the same temperature as the hydrophobic phase and added dropwise into the non-aqueous phase under magnetic stirring. The consequential preparation was stirred for another 10 min, and then ultrasonication was done. Then, the preparation placed on an ice bath and diluted to a desired volume. Finally, the preparation was filtered through a 0.22 µm membrane filter [12].

Both the preparations were centrifuged separately. The formed pellet was washed with sterile double distilled deionised water and re-centrifuged; this step was repeated four times and the pellet then re-suspended in an appropriate amount of sterile double distilled deionised water.

For chitosan-coated nanoliposomes, an appropriate amount of percentage (w/v) chitosan solution was dropped into the nanoliposomal suspension under magnetic stirring at room temperature. Then, the resulting solution was stirred for approximately 1 h and then incubated overnight at 4 ºC [5, 13].

% Encapsulation Efficiency: The release experiments were run immediately after the separation of the free geftinib from that

encapsulated in nanoliposomes. The nanoliposomes and chitosan-coated nanoliposomes shells are lysed completely by adding alcohol and doing sonication. Then the contents were assayed by High-Performance Liquid Chromatography (HPLC) in triplicate at 254 nm wavelength. The analysis of samples was done by using HPLC Agilent technologies 1200 series, Germany; Quaternary pump, Eclipse XDB- C18 column (4.6 mm × 150 mm) which is filled with octadecylsilane bonded to porous silica (3 µm). The acetonitrile and 1% ammonium acetate in ratio of 3:2 was used as a mobile phase which was flowing at the rate of 1 ml/min at 25 ºC for analysis [14].

The following equation was used to compute the % encapsulation efficiency of gefitinib nano-liposomes:

$$\underset{\text{Efficiency}}{\%\ \text{Encapsulation}} = \frac{\text{Total drug} - \text{free drug}}{\text{Total drug}} \times 100$$

Transmission Electron Microscopy: Nano-liposomes and chitosan-coated nanoliposomes were analysed using a transmission electron microscope (TEM) to describe the detailed structure morphology of the nanoliposomes. A drop of each uncoated and chitosan coated nanoliposomal suspension was added into copper coated carbon grid through micropipette separately. The filter paper was used to remove the excess sample and then grid was placed to examine under the electron microscope. The diameters of the nanoliposomes were determined using the scale bar on the electron micrographs [15].

Particle Size and Zeta Potential Measurements: Zeta potential of uncoated and chitosan-coated nanoliposomes with different chitosan percentages (0.01 - 0.5%, w/v) was estimated using the Malvern Zetasizer, Nano ZS90 (Malvern Instruments, U.K.). Pellets of nanoliposomes were perched in double distilled deionised water. To confirm the coating on the outer surface of particle, particle size and surface charge on the chitosan-coated nano-liposomes was calculated after centrifugation.

Fourier Transforms Infrared Measurements: Instrument FT-IR-Alpha Bruker 1206 0280, from Germany was used to accomplish the structural depiction of prepared nanocarter systems and raw materials. FT-IR spectra of soya lecithin, formulation containing uncoated nanoliposomes, cholesterol, gefitinib, chitosan, formulation containing chitosan coated nanoliposomes were obtained from wave number 4000c m^{-1} to 400 cm^{-1}. In IR spectroscopy, first of all background measurement was done to nullify the effect of environmental impurities in spectra[16]. The overlay of above spectras was shown in **Fig. 4**.

Differential Scanning Calorimetric Analysis (DSC): Instrument DSC Q10 (Waters Corporation, USA) was used for DSC measurements to predict the melting point. The calibration of apparatus was done with indium. The samples were converted into pellets by using aluminium pans for analysis and then the temperature was gradually rises at the rate of 10 °C/min from 25 °C to 300 °C in nitrogen atmosphere. The nitrogen was flow at the rate of 60 ml/min. Under same conditions, for reference an empty pan was used [17].

Physicochemical Stability of Nanocarrier System: To determine the stability of the uncoated and chitosan-coated nanoliposomes coated with 0.01%, w/v chitosan solution, these samples were stored at freeze temperature (4 ºC ± 1 ºC), room temperature (25 ºC ± 2 ºC/ 60% ±5% RH), and 45ºC ± 2 ºC/ 75% ± 5% RH (as per ICH guidelines). Then, their particle size, % encapsulation efficiency, viscosity, surface tension, physical appearance and ζ-potential were estimated for three months [18, 19].

***In-vitro* Release of Gefitinib from Nano-liposomes:** The dialysis bag method was used to study the *in-vitro* release profile of gefitinib. The persistent release was scrutinized in three different media like the simulated gastric juice (containing 0.1 N HCl and pepsin enzyme), acetate buffer (pH 4.0) and intestinal juice (pH 6.8) containing trypsin enzyme to study the release of drug in these media.

For this volume of uncoated and coated gefitinib nanoliposomes suspension equivalent to 250 mg of gefitinib was taken into the dialysis bag (molecular weight cutoff, 12000 Da, Himedia, Mumbai, India) and sealed at both ends. The dialysis bag was dipped in the cylindrical vessel of USP dissolution apparatus II (Lab India DS 8000, Mumbai, India); containing 200 ml of dissolution media maintained at a temperature of 37 ºC ± 0.5 ºC.

The rotating speed was set at 100 rpm. At preset intervals (0.5, 1, 2, 4, 6, 8, 12, 18, 24) a predetermined volume (2 ml) was replaced by fresh preheated media. The sample was analysed after filteration through 0.45 µm filter through HPLC. All the experiments were performed in triplicate and the average values were determined [20, 21].

Data Analysis: Results are expressed as mean ± standard deviation of the outcomes. The results were calculated using microsoft Excel software from at least three experiments [22]. $p<0.05$ value was used to indicate the level of significance.

RESULTS AND DISCUSSION:

Formation of the Nanocarter System: In this research, the sustainable nanocarter systems were prepared by reverse-phase evaporation method and modified emulsification and ultrasonication method. Chitosan was used as a coating material to coat the plain nanoliposomes. The physicochemical characters and *in-vitro* release profile of encapsulated material of the prepared nanocarter systems could be affected by varied concentrations of chitosan. Initially, liposomes are formed in both methods and then ultrasonication was done to reduce their size to nanorange by creation and burst of various bubbles formed due to ultrasonication. Then, the samples were analysed for their particle size and zeta potential. The coating of nano-liposomes by chitosan had shown a minor increase in the size of the nanoliposomes, signifying the development of coating layer on the nanoliposomes surface. The uncoated particles were carried negative charge due to ionization of carboxyl groups of fatty acid moieties and soya lecithin. The results of zeta potential studies demonstrated that the surface charge was altered reversely. Initially, the nanoliposomes had negative charge which was converted into positive charge after coating with chitosan. In acidic medium the chitosan bears a positive charge due to presence of free amino group as $-NH^{3+}$. Due to ion-ion interaction between these free amino groups and the negatively charged surface of nanoliposomes the surface charge of particles amend from negative to positive after the coating process [22].

Effect on Morphology: Surface morphological studies of chitosan coated nanoliposomes and uncoated nanoliposomes were visualized using transmission electron microscopy. The images demonstrated that the particles in formulations were nearly spherical in shape **Fig. 1**. Further, in TEM images of chitosan-coated nanoliposomes the chitosan coating on the surface of nanoliposomes was clearly observed **Fig. 1 C** and **D**.

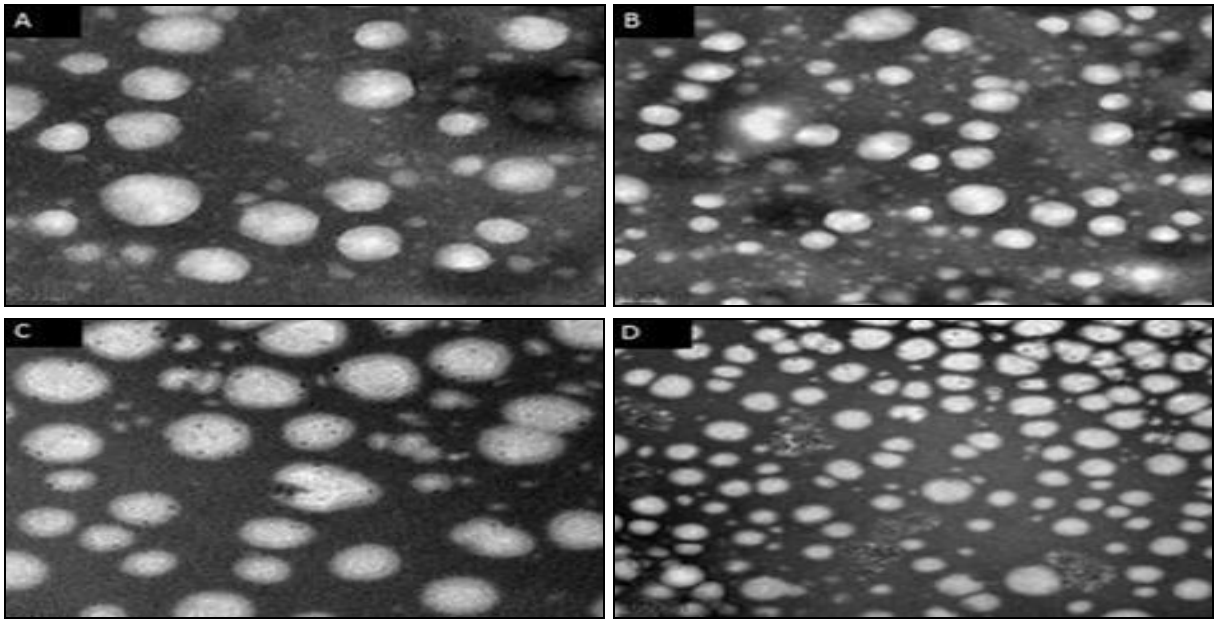

FIG. 1: TRANSMISSION ELECTRON MICROGRAPHS OF (A) UNCOATED NANOLIPOSOMES PREPARED BY REVERSE-PHASE EVAPORATION METHOD. BAR IS 50 nm, (B) UNCOATED NANOLIPOSOMES PREPARED BY MODIFIED EMULSIFICATION AND ULTRASONICATION METHOD. BAR IS 100 nm, (C) CHITOSAN-COATED NANOLIPOSOMES PREPARED BY REVERSE-PHASE EVAPORATION METHOD. BAR IS 50 nm, (D) CHITOSAN-COATED NANOLIPOSOMES PREPARED BY MODIFIED EMULSIFICATION AND ULTRASONICATION METHOD. BAR IS 100 nm

Effect on Zeta Potential: The outcomes of different percentages of chitosan solution on the zeta potential of the nanoliposomes are also shown in **Fig. 2A** and **B**. Initially, the nanoliposomes had negative charge of phosphate (PO_4^{3-}) groups due to ionization of fatty acid moieties and soya lecithin [23]. The chitosan molecule carried a positive charge due to free amino groups in its structure and due to its positive charge, it attracted easily towards the negatively charged structure of nanoliposomes. Thus, the ionic magnetism between these free amino groups and the negatively charged surface of nanoliposomes modified the exterior charge of particles reversely from negative to positive after the coating process. This amendment of the surface charge of the nanoliposomes was the confirmation of chitosan covering on the exterior of the nanoliposomes. Chitosan, due to a higher inherent ζ-potential value, chitosan could contribute a relatively high ζ-potential value to the resulting nanocarter systems. It is generally said that the increased concentration of chitosan raised the intrinsic surface charge due to increase in ionic interaction between the surface of nanoliposomes and chitosan molecules. We used different concentrations of chitosan like 0.01- 0.5% (w/v), and found that as the percentage of chitosan solution increased, the value of ζ-potential was also boost up, before coming to a relatively constant value in both cases.

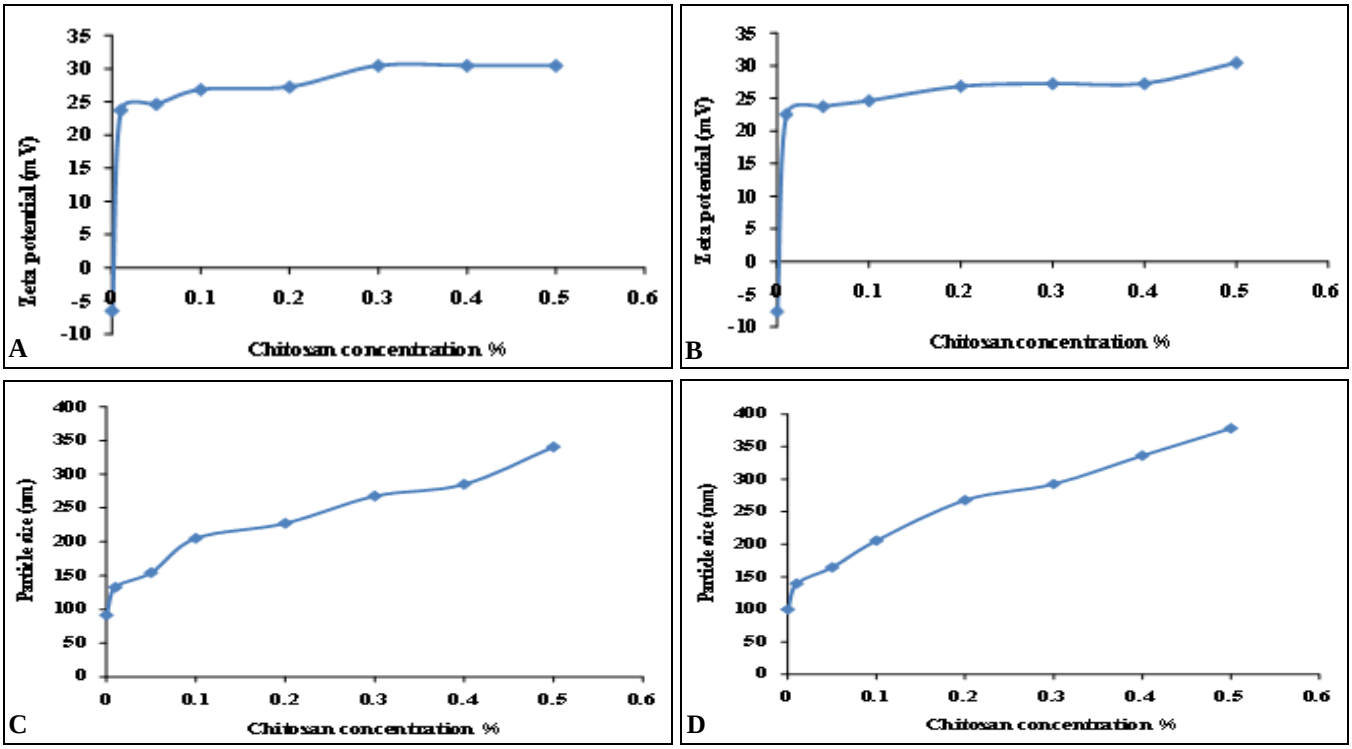

FIG. 2: (A) EFFECT ON ZETA POTENTIAL OF NANOLIPOSOMES PREPARED BY REVERSE-PHASE EVAPORATION METHOD WITH THE CHANGE IN CONCENTRATION OF CHITOSAN AND (B) EFFECT ON ZETA POTENTIAL OF NANOLIPOSOMES PREPARED BY MODIFIED EMULSIFICATION AND ULTRASONICATION METHOD WITH THE CHANGE IN CONCENTRATION OF CHITOSAN, (C) EFFECT ON PARTICLE SIZE OF NANOLIPOSOMES PREPARED BY REVERSE-PHASE EVAPORATION METHOD WITH THE CHANGE IN CONCENTRATION OF CHITOSAN, (D) EFFECT ON PARTICLE SIZE OF NANOLIPOSOMES PREPARED BY MODIFIED EMULSIFICATION AND ULTRASONICATION METHOD WITH THE CHANGE IN CONCENTRATION OF CHITOSAN

Effect on Particle Size: The achieves of different percentages of chitosan solutions on the size of the nanoliposomes is shown in **Fig. 2C, D**. The coating of nanoliposomes by chitosan had shown a marginal increase in the size of the nanoliposomes, signifying the development of veneer layer on the nanoliposomes exterior. As the percentage of chitosan solution increases, the thickness of covering layer on the exterior of nanoliposomes was also increased, resulted in an increased in diameter of nanoliposomes. The small modification in particle size is enviable for systemic circulation, since, the cells of the mononuclear phagocyte system tend to clear the big particles from the

circulation [24]. The strong intra-molecular electrostatic force of attraction leads to the formation of fine layer of chitosan solution on the exterior of nanoliposomes. The opposite charge of fragments of the polyelectrolyte chains and groups situated on the exterior of nanoliposomes compel them to come in propinquity and form a lean layer on the exterior of nanoliposomes. T-Test was made for the value of size of nanoliposomes before and after chitosan coating. Less than 0.01 P-value was indicated that the difference in size between uncoated and chitosan coated nanoliposomes was highly significant. The combination of adsorption coagulation and bridging between chitosan and nanoliposomes surface appears to be responsible for interaction between them [18].

Fourier Transforms Infrared Measurements: In IR spectroscopy, first of all background measurement was done to nullify the effect of environmental impurities in spectra and then spectra were recorded from wave number 4000cm^{-1} to 400 cm^{-1}. The overlay of soya lecithin, formulation containing uncoated nanoliposomes, cholesterol, gefitinib, chitosan, formulation containing chitosan coated nanoliposomes spectra was shown in **Fig. 3**.

In FTIR spectroscopy the bandwidth and the frequency of different functional groups was analysed to examine even slight modifications in the arrangement of the lipid assemblies. The head-group region of acyl chains and of lipid molecules was also investigated to study the detail about the arrangement of lipid molecules in the absence or presence of chitosan. In the chitosan spectra (e), the band for carbonyl amide (amide I) was found in range 1637.39 - 1419.23 cm^{-1}. The band in range 1063.51-1027.67 cm^{-1} almost matches to the –COC groups symmetric stretching of chitosan molecule. These peaks were also observed in FTIR spectra of chitosan-coated nanoliposomes (f). But these peaks were not visible in the FT-IR spectra of uncoated nanoliposomes (b). The sturdy and broad peak in the range of 3500 - 3300 cm^{-1} almost corresponds to hydrogen-bonded stretching vibration and N-H stretching from primary amine. The peak in the range of 2923.28 - 2850.91 cm^{-1} was represented the -CH stretching.

Differential Scanning Calorimetric Analysis (DSC): DSC analysis was used as a method for the confirmation of drug. The DSC analysis had shown a pointed endothermic peak at 197.51 °C almost corresponds to the melting point range *i.e.* 193 - 198 °C of drug with percentage purity 99.42 mol % **Fig. 4**.

A pointed endothermic peak at 153.87 °C attributed to the melting point of cholesterol. The desertion of drug peak in analysis of formulation indicates that during formation of the nanoliposomes the drug may either disperse or dissolve in the polymer medium. The whole drug incorporation into the nanoliposomes recommended a molecular dispersal of the drug within the system. A pointed endothermic peak almost corresponds to the melting point range of chitosan indicates that the nanoliposomes were coated with chitosan particles.

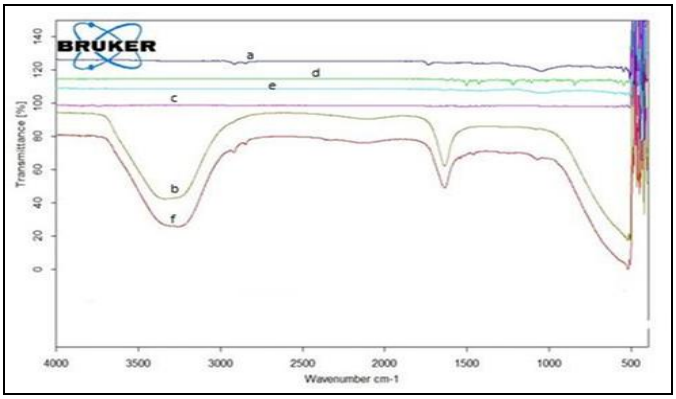

FIG. 3: OVERLAY OF FTIR SPECTRAS OF (A) SOYA LECITHIN (B) FORMULATION CONTAINING UNCOATED NANOLIPOSOMES (C) CHOLESTROL (D) GEFITINIB (E) CHITOSAN (F) FORMULATION CONTAINING COATED NANOLIPOSOMES

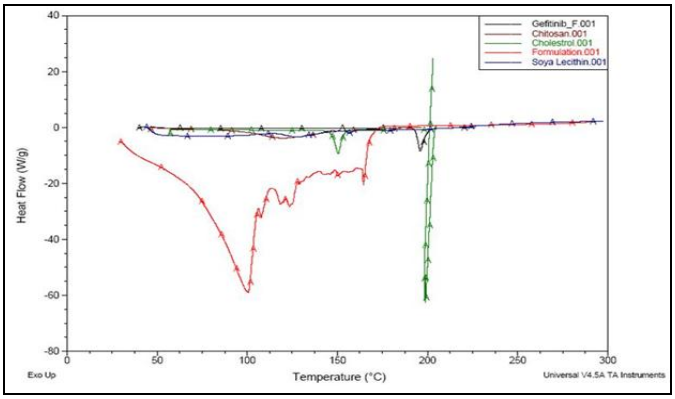

FIG. 4: OVERLAY OF DSC ANALYSIS OF GEFITINIB, CHITOSAN, CHOLESTEROL, FORMULATION, SOYA LECITHIN

Stability Studies of Nanocarter System: The steadiness of nanoliposomes is another important factor to be considered in the development of an efficient drug delivery system. Therefore, we evaluated the stability of both uncoated and chitosan coated nanliposomal formulations at different temperatures, to imitate physiological conditions. The results obtained have shown that the prepared vesicles were physically and chemically stable at 4 °C for 3 months. During the time period of stability study, the samples stored at 4 °C did not show any momentous modification in the particle size, % encapsulation efficiency, physical appearance, and zeta potential for both the chitosan coated nanoformulations. But the samples hoard at 25 °C and 45 °C produced a marked increase in particle size both in uncoated and chitosan coated nanoformulations, which may be due to the aggregation or swelling of nanoliposomes. A significant decrease in the encapsulation efficiency was observed which shows a significant leakage of gefitinib from both the formulations over time.

The physical appearance of both the uncoated and chitosan coated nanoliposomal formulations hoard at 4 °C, 25 °C, and 45 °C for 1, 2, and 3 months was estimated. After 1st month, the entire samples hoard at 4 °C and 25 °C were found to be secured. But the samples placed at 45 °C, had revealed alteration in the tint, surface tension and viscosity of samples with small clusters of particles but they were dispersible on shaking.

The mean particle size and the zeta potential of both the formulations as a function of temperature and time were also evaluated. Small spots of fungus growth was observed on top of the all the formulation stored at 45 °C at the end of 2nd month, which led to discontinuation of the stability studies at 45 °C after 2 month. Except for the nano-liposomes stored at 45 °C, which led to discontinuation of the stability studies at 45 °C after 2 month, there is no significant change in the particle size and zeta potential of other samples hoard at 4 °C and 25 °C. At the end of 3rd month, the entire samples hoard at 25 °C also showed fungal growth, while those at 4 °C were stable.

Encapsulation efficiency of the uncoated nanoliposomes prepared by REV method and MEU method stored at 4 °C decreased by 12, 21, 31% and 10, 19, 32%, whereas those stored at 25°C decreased by 18, 38, 57% and 19, 34, 60%, while those stored at 45 °C decreased by 46, 66, 81% and 43, 66, 81% at the end of 1, 2 and 3 months respectively. For the chitosan coated nano-liposomes stored at 4 °C, encapsulation efficiency decreased by 6, 9, 20% and 5, 10, 21%, whereas for those stored at 25 °C decreased by 14, 30, 64% and 11, 32, 65%, while those stored at 45°C decreased by 43, 63, 81% and 41, 62, 83% at the end of 1, 2, and 3 months, respectively. The results of particle size, zeta potential, surface tension, viscosity, physical appearance and % EE of both the formulation over the span of 3 months were shown in **Table 1 - 4**.

TABLE 1: STABILITY STUDIES OF UNCOATED AND CHITOSAN COATED NANOLIPOSOMES AT 0 MONTH (B) 1ST MONTH (M1 = UNCOATED NANOLIPOSOMES PREPARED BY MEU METHOD, M2 = CHITOSAN COATED NANOLIPOSOMES PREPARED BY MEU METHOD, R1= UNCOATED NANOLIPOSOMES PREPARED BY REV METHOD, R2 = CHITOSAN COATED NANOLIPOSOMES PREPARED BY REV METHOD)

Temperatures	**4 ºC**				**25 ºC**				**45 ºC**			
Parameters	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**
Particle size (nm)	99.88 ±3.07	139.5 ±4.67	93.2 ±3.25	132. 7±4.56	99.88 ±3.07	139.5 ±4.67	93.2 ±3.25	132.7 ±4.56	99.88 ±3.07	139.5 ±4.67	93.2 ±3.25	132.7 ±4.56
Zeta potential (mV)	-7.68 ±0.02	22.6 ±0.34	-6.51 ±0.03	23.7 ±0.03	-7.68 ±0.02	22.6 ±0.34	-6.51 ±0.03	23.7 ±0.03	-7.68 ±0.02	22.6 ±0.34	-6.51 ±0.03	23.7 ±0.03
% EE	88.91 ±0.67	88.7 ±0.78	87.56 ±0.17	88.46 ±0.97	88.91 ±0.67	88.7 ±0.78	87.56 ±0.17	88.46 ±0.97	88.91 ±0.67	88.7 ±0.78	87.56 ±0.17	88.46 ±0.97
Surface tension (dynes/cm)	38.49 ±0.65	43.46 ±0.37	42.01 ±0.32	42.9 ±0.43	38.49 ±0.65	43.46 ±0.37	42.01 ±0.32	42.9 ±0.43	38.49 ±0.65	43.46 ±0.37	42.01 ±0.32	42.9 ±0.43
Viscosity (cP)	1.23 ±0.02	1.25 ±0.03	1.07 ±0.01	1.17 ±0.02	1.23 ±0.02	1.25 ±0.03	1.07 ±0.01	1.17 ±0.02	1.23 ±0.02	1.25 ±0.03	1.07 ±0.01	1.17 ±0.02
Physical appearance	Clear				Clear				Clear			

TABLE 2: STABILITY STUDIES OF UNCOATED AND CHITOSAN COATED NANOLIPOSOMES AT 1ST MONTH (M1= UNCOATED NANOLIPOSOMES PREPARED BY MEU METHOD, M2= CHITOSAN COATED NANOLIPOSOMES PREPARED BY MEU METHOD, R1= UNCOATED NANOLIPOSOMES PREPARED BY REV METHOD, R2= CHITOSAN COATED NANOLIPOSOMES PREPARED BY REV METHOD)

Temperatures	4 ºC				25 ºC				45 ºC			
Parameters	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**
Particle size (nm)	106.4 ±4.09	142.7± 4.89	128.7 ±3.06	139.5 ±4.65	294.5 ±3.89	228.4 ±4.67	279.5 ±3.09	212.2 ±4.89	620.2 ±7.89	489.5 ±6.75	528.3 ±5.45	440.8 ±7.23
Zeta potential (mV)	-7.45 ±0.03	22.4 ±0.03	-6.46 ±0.05	23.4 ±0.02	-5.67 ±0.03	19.6 ±0.05	-5.23 ±0.03	20.6 ±0.06	-3.67 ±0.04	11.5 ± 0.02	-3.45 ±0.04	12.9 ±0.03
% EE	80.71 ±0.75	84.27± 0.87	78.75 ±0.56	83.69 ±0.65	72.36 ±1.09	78.76 ±2.34	73.46 ±1.23	76.96 ±2.39	50.37 ±3.23	52.27 ±3.24	48.27 ±4.34	50.79 ±4.35
Surface tension (dynes/cm)	42.67 ±0.23	42.54± 0.91	43.105 ±0.46	40.35 ±0.34	60.01 ±0.56	63.9 ±0.46	54.9 ±0.43	57.1 ±0.36	76.37 ±0.47	70.76 ±0.67	68.96 ±0.58	65.86 ±0.69
Viscosity (cP)	1.6 ±0.01	1.45 ±0.012	1.34 ±0.34	1.29 ±0.23	3.89 ±0.34	2.87 ±0.67	3.89 ±0.23	2.83 ±0.56	9.75 ±0.89	9.5 ±0.29	9.99 ±0.98	9.67 ±0.67
Physical appearance	Translucent				Milky				Pale yellow with small clusters of particles dispersed on shaking			

TABLE 3: STABILITY STUDIES OF UNCOATED AND CHITOSAN COATED NANOLIPOSOMES AT 2ND MONTH (M1 = UNCOATED NANOLIPOSOMES PREPARED BY MEU METHOD, M2 = CHITOSAN COATED NANOLIPOSOMES PREPARED BY MEU METHOD, R1 = UNCOATED NANOLIPOSOMES PREPARED BY REV METHOD, R2 = CHITOSAN COATED NANOLIPOSOMES PREPARED BY REV METHOD)

Temperatures	4 ºC				25 ºC				45 ºC			
Parameters	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**
Particle size (nm)	170.4 ±3.89	161.3 ±4.68	148.2 ±4.89	153.5 ±5.67	348.9 ±6.75	335.9 ±4.35	345.5 ±3.89	309.5 ±4.96	9054 ±3.16	7834 ±3.56	8898 ±4.45	7366 ±4.12
Zeta potential (mV)	-7.25 ±0.01	22.06 ±0.03	-6.23 ±0.01	22.5 ±0.05	-4.32 ±0.06	14.3 ±0.07	-4.67 ±0.03	15.7 ±0.03	-2.01 ±0.03	4.2 ±0.05	-2.67 ±0.03	5.9 ±0.02
% EE	72.76 ±2.34	80.06 ±2.67	70.36 ±2.98	81.23 ±1.64	58.36 ±1.56	60.47 ±1.96	55.46 ±2.45	61.76 ±2.09	30.27 ±3.12	33.47 ±2.09	30.02 ±2.15	32.46 ±1.98
Surface tension (dynes/cm)	55.8 ±0.98	55.4 ±0.87	54.2 ±0.56	53.8 ±0.78	73.26 ±0.56	72.89 ±0.67	72.6 ±0.89	72.07 ±0.67	94.76 ±0.78	94.25 ±0.35	94.25 ±0.46	94.16 ±0.57
Viscosity (cP)	1.8 ±0.89	1.69 ±0.67	1.85 ±0.23	1.79 ±0.45	5.79 ±0.67	5.56 ±0.12	5.45 ±0.67	5.32 ±0.56	15.75 ±0.23	14.26 ±0.45	15.26 ±0.56	14.07 ±0.45
Physical appearance	Milky				Pale yellow with small clusters of particles dispersed on shaking				Dark colour suspension with fungus clumps do not dispersed on shaking			

TABLE 4: STABILITY STUDIES OF UNCOATED AND CHITOSAN COATED NANOLIPOSOMES AT 3RD MONTH (M1 = UNCOATED NANOLIPOSOMES PREPARED BY MEU METHOD, M2 = CHITOSAN COATED NANOLIPOSOMES PREPARED BY MEU METHOD, R1 = UNCOATED NANOLIPOSOMES PREPARED BY REV METHOD, R2 = CHITOSAN COATED NANOLIPOSOMES PREPARED BY REV METHOD)

Temperatures	4 ºC				25 ºC				45 ºC			
Parameters	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**	**M1**	**M2**	**R1**	**R2**
Particle size (nm)	248.8 ±6.75	192 ±5.98	220.8 ±4.89	172.6 ±5.02	1810 ±7.84	1341 ±7.89	1772 ±10.23	1211 ±12.23	-	-	-	-
Zeta potential (mV)	-7.02 ±0.04	21.9 ±0.06	-6.02 ±0.05	22.07 ±0.07	-2.89 ±0.03	11.7 ±0.08	-2.78 ±0.09	12.6 ± 0.05	-	-	-	-
% EE	60.76 ±4.56	71.35 ±3.46	61.75 ±4.67	70.56 ±5.23	35.65 ±4.67	38.43 ±5.75	31.27 ±3.45	32.36 ±2.34	16.65 ±1.56	16.76 ±1.98	15.36 ±2.78	16.79 ±3.89
Surface tension (dynes/cm)	58.9 ±1.02	60.5 ±0.98	62.8 ±0.78	60.4 ±0.76	79.78 ±0.45	78.56 ±0.67	80.97 ±0.89	77.02 ±0.87	99.78 ±1.08	98.23 ±1.07	99.99 ±1.41	96.78 ±0.79
Viscosity (cP)	2.4 ±0.12	2.29 ±0.23	2.45 ±0.45	2.35 ±0.23	6.59 ±0.89	5.76 ±0.78	6.45 ±0.78	5.52 ±0.97	19.35 ±0.56	18.86 ±0.67	19.76 ±0.56	17.97 ±0.45
Physical appearance	Milky to slightly pale yellow				Dark colour formulation with fungus clumps dispersed on shaking				Dark colour formulation with fungus clumps do not dispersed on shaking			

During 1st month, all samples containing chitosan-coated nanoliposomes did not show any momentous modification in particle size, % encapsulation efficiency, viscosity, surface tension and zeta potential. Moreover, a considerable outcome on particle dimensions was noticed after 2 months. These results either may be due to swelling of outer layer of nanocarter systems or may be due to alteration in their surface properties. Alternatively, uncoated nanoliposomes began to

point up considerable amendments in size just after 1 month. This diversity in stability among the chitosan-coated and uncoated nanoliposomes may be to the chitosan covering, its multilayer wall can impede the distension and release of entrapped material. Thus, the plain nanoliposomes become more stable due to chitosan coating. Therefore, through the use of various percentages of chitosan solution as coating material, we can prepare an appropriate sustained drug release chitosan-coated nanoliposomes.

***In-vitro* Release: Fig. 5** shows the effect of chitosan coating on gefitinib nanoliposomes on the drug release rate from nanoliposomes at different time intervals. It is clear that the percentage increase of drug release from nanoliposomes was reduced after coating with chitosan at all examined time intervals in all three media. For example, after 4 h, the percentage of drug release was found to be 30% and less than 28% for gefitinib nanoliposomes and chitosan-coated nanoliposomes respectively.

The protective effect of hydrophilic polymer coating depends on the adhesion of the polymer to the lipid molecule [5]. Mady *et al.*, [26] observed that the interaction between chitosan and nano-liposomes enhanced the stability of lipid vesicles. As the percentage of chitosan solutions increases the intrinsic surface charge and the thickness of outer coating on the nanocarter system along with the sustainable release period of entrapped material from the nanocarter system was also prolonged.

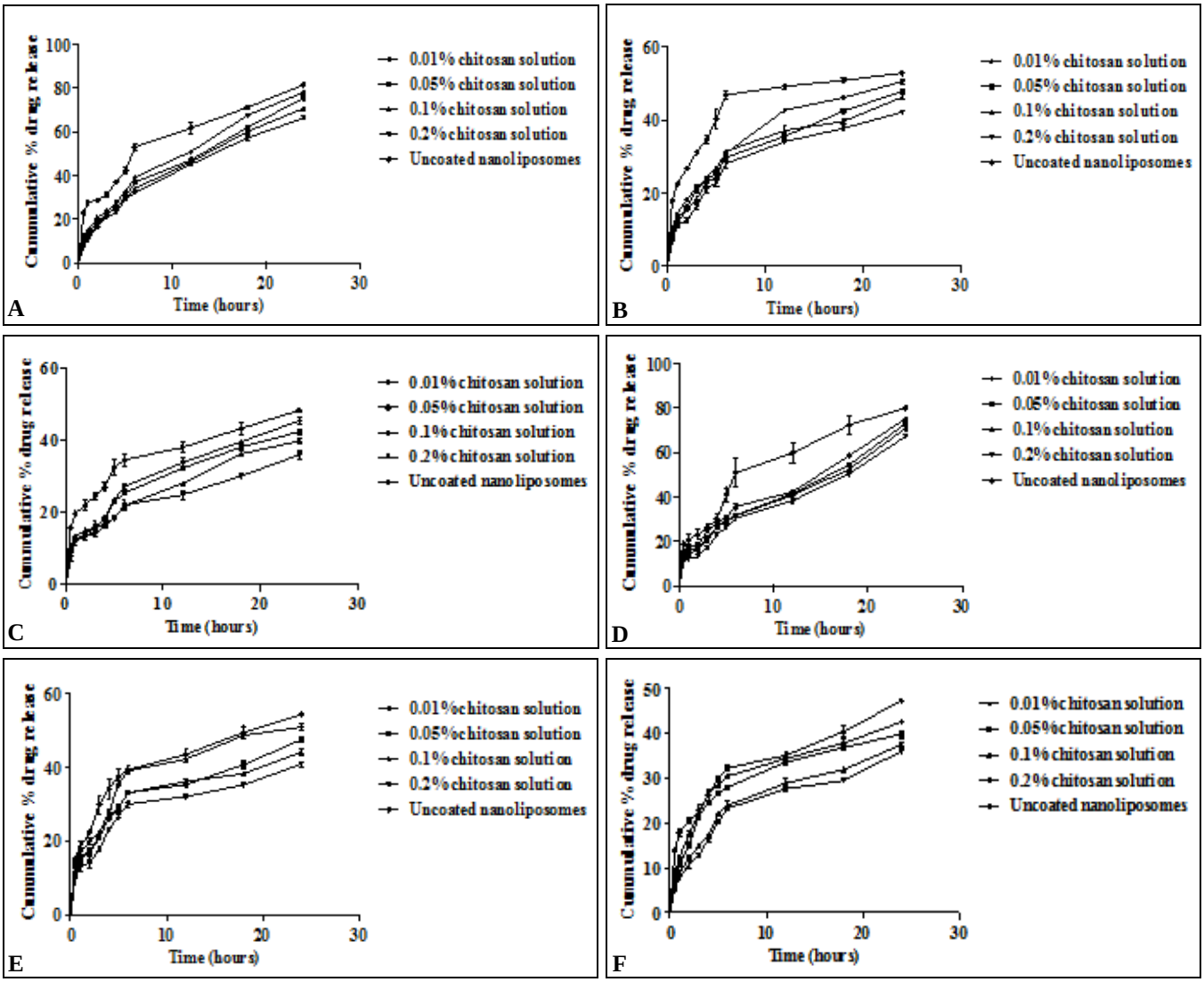

FIG. 5: *IN VITRO* RELEASE OF DRUG FROM UNCOATED AND CHITOSAN COATED NANOLIPOSOMES PREPARED BY REV (A, B, C) AND MEU (D, E, F) METHOD. Values are expressed as mean ± S.D.

Fig. 5 shows the *in-vitro* release profiles of entrapped material from uncoated and chitosan coated nanoliposomes in the simulated gastric juice (pH 1.3) (A, D), acetate buffer (pH 4.0) (B, E) and intestinal juice (pH 6.8) (C, F). The significantly slow release of entrapped material from nanoliposomes indicated their depository effect ($p<0.01$). The kinetic study of *in-vitro* release data demonstrated that the release profiles from all nanoliposomal samples followed first order release kinetic and log of time dependent Korsemeyer Peppas Plot model, which is illustrated as Fickian type diffusion.

These results specified that the gefitinib liberation from the nanoliposomes appeared to be restricted by solvent penetration, polymer chain disentanglement, swelling, and relaxation procedures. In chitosan coated nanoliposomes, the release of entrapped material was slow down demonstrating the reservoir effect of nanoliposomes. The sustained release behavior of chitosan coated nanoliposomes was due to chitosan coating and the stability of chitosan coated nanoliposomes in serum. This is perhaps due to formation of multilayer wall on the exterior of nanoliposomes which restricts the fluidity of lipid bilayer and decreases the membrane permeability [27].

Cytotoxicity Studies: Cytotoxicity studies of pure drug gefitinib, uncoated and chitosan coated nanoliposomes were determined in A549 and H1299 cells by the MTT assay. The IC_{50} values for free gefitinib, uncoated and chitosan coated nanoliposomes in A549 cells and H1299 cells are summarized in **Table 5**.

TABLE 5: CYTOTOXICITY OF GEFITINIB LOADED NANOLIPOSOMAL FORMULATIONS TO A549 AND H1299 CELLS RESPECTIVELY. CYTOTOXICITY WAS DETERMINED USING MTT ASSAY AS DESCRIBED IN THE MATERIALS AND METHODS

Sample Codes	A549 Cells (IC_{50}; µM)	H1299 Cells ($1C_{50}$; µM)
M1	9.32±1.25	8.54±1.08
M2	7.98± 0.97	5.07±0.87
R1	7.31±0.85	7.03±0.98
R2	6.53±0.75	4.73±0.46
Gefitinib	1.06±0.7	1.92±0.86

Data represent the mean ± SD (n=3)

In A549 and H1299 cells, free gefitinib led to the highest toxicity. In contrast, uncoated and chitosan coated nanoliposomes had reduced cytotoxicity in A549 and H1299 cells respectively. Free gefitinib had shown more cytotoxicity than uncoated and chitosan coated nanoliposomes, representing stable confiscation of gefitinib by nanoliposomal encapsulation. The less cytotoxicity of uncoated and chitosan coated nanoliposomes might be due to the slow release rate of gefitinib from the nanoliposomes. The IC_{50} values for free gefitinib were 1.06 ± 0.7 and 1.92 ± 0.86 µM respectively in A549 and H1299 cells after incubation, while the IC_{50} values for uncoated nanoliposomes prepared by REV method and MEU method in A549 and H1299 cells were 7.31 ± 0.85, 9.32 ± 1.25 and 7.03 ± 0.98, 8.54 ± 1.08 µM respectively. These results indicated that free gefitinib was found to be more cytotoxic than uncoated and chitosan coated nanoliposomal formulation in the cell culture system.

However, chitosan coated nanoliposomes had shown a marginally improved inhibitory potential as compared to uncoated delivery system. The IC_{50} values for chitosan coated nanoliposomes prepared by REV method and MEU method in A549 and H1299 cells were 6.53 ± 0.75, 7.98 ± 0.97 and 4.73 ± 0.46, 5.07 ± 0.87 µM respectively. In the present study, the cytotoxicity of chitosan coated nanoliposomal formulation might be associated with the zeta potential of the prepared nanoliposomes. As the chitosan coated (M2 and R2) formulations (22.6 ± 0.34 mV, 23.7 ± 0.03mV) have more zeta potential than uncoated (M1 and R1) formulations (-7.68 ± 0.02 mV, -6.51 ± 0.03 mV) so the % inhibition of the A549 and H1299 cells was found more in these formulations. Higher value of positive zeta potential facilitates cytotoxicity in cancer cells due to stronger interaction with tumor cell membrane [28, 29]. He *et al.,* found that an increase in zeta-potential values of carboxymethyl chitosan grafted methyl methacrylate nanoparticles resulted in a significant rise in the cellular uptake of nanoparticles in murine macrophage cells [29].

Among chitosan coated nanoliposomes, the nanoliposomes prepared by REV method had shown more cytotoxicity than nanoliposomes prepared by MEU method the batch R2 has shown more cytotoxicity than batch M2 in both A549 and H1299 cell lines. The particle size of chitosan coated nanoliposomes appeared to be the

controlling factor affecting the cytotoxicity of the batch M2. A decrease in the size of nanoliposomes may lead to an increase in the interactions and binding with cell membranes, which can result in enhanced cytotoxicity of chitosan coated nano-liposomes [28, 30, 31]. Zhang *et al*., reported that drug uptake in cancer cells reliant on the particle size. Particle size of nanocarriers has been reported to play a substantial role in their antitumor activity. Thus, cytotoxic activity of chitosan coated nano-liposomes increased by using nanoliposomes with smaller particle size [32]. Ha *et al*., demonstrated that the size and surface charge of β-lg nanoparticles were play an important role in the cellular uptake of β-lg nanoparticles in Caco-2 cells [28].

CONCLUSION: In this research, the coating process was used to prepare a sustainable nanocarter system. The plain nanoliposomes prepared by REV and MEU methods were coated with different concentrations of chitosan. These experiments were performed with diverse percentages of chitosan solutions under similar conditions. The optimized coating concentration was the 0.01% (w/v) for both nanocarter systems prepared by both REV and MEU methods. The accurate experimental conditions to obtain the desired particle size and absolute value of zeta potential were determined. Different techniques like transmission electron microscope images, FTIR studies, particle size and the zeta potential analysis was done to confirm the coating on the exterior of gefitinib nanoliposomes. The coating on vesicles is clearly observable in these micrographs.

Chitosan coating resulted in buildup of particle size and alteration in surface charge from negative to positive value on nanoliposomes which results in a more stable nanosystem. Chitosan coating has a significant effect on drug release behavior. The sustained release of entrapped material from the nanocarter system in all media was the most momentous result. Different percentages of chitosan solutions were used in a release test to estimate the optimum percentage for sustained release of entrapped drug. As the percentage of the coating material increased, the thickness of coating layer also raised and the release of entrapped core material became lengthy. Thus, the results signified that diverse amount of chitosan could influence the physicochemical and release characters of the resulting nanocarter systems. The results from stability studies also demonstrated that the chitosan coated nanoliposomal formulation is found to be more stable at 4 ºC. The cell lines studies demonstrated that the chitosan coated nano-liposomes had shown better potency than uncoated nanoliposomes. Suitable amalgamation of the nano-liposomes and chitosan characters may produce nanoliposomes with desired, extended and sustained release behavior.

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