# Synopsis

of the thesis entitled

"Delivery of siRNA for masking resistance to chemotherapy in non-small cell lung cancer"

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**DOCTOR OF PHILOSOPHY**

**IN**

**PHARMACY**

By

**Mr. Vivek N. Patel**

M.Pharm

Supervised By

**Prof Ambikanandan Misra**

Professor,

Pharmacy Department, Faculty of Pharmacy

Faculty of Pharmacy

The Maharaja Sayajirao University of Baroda

Vadodara -390001, Gujarat, India

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# Synopsis

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| Name of the PhD Research Scholar: | Mr. Vivek N. Patel |
| Registration number: | FOPH/2 |
| Registration date: | 20/05/2016 |
| Title: | Delivery of siRNA for Masking Resistance to Chemotherapy in Non-small Cell Lung Cancer |
| Name and Designation of Research Guide: | Prof Ambikanandan Misra, Professor, Faculty of Pharmacy, The M. S. University. |
| Department: | Pharmacy Department |
| Institute: | Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda. |

| | |
|---|---|
| Mr. Vivek N. Patel | Prof. Ambikanandan Misra |
| PhD Research Scholar | Research Guide |

| | |
|---|---|
| Head | Dean, |
| Pharmacy Department, | Faculty of Pharmacy |
| Faculty of Pharmacy | The Maharaja Sayajirao University of Baroda |

# Contents

# 1. Introduction

The collaborative research is proposed for evaluating effectiveness of silencing a gene that confers resistance to chemotherapeutic agents in treatment of non-small cell lung cancer (NSCLC) using hybrid lipo-polymeric vesicles (HLPs) as a novel non-viral vector comprising of lipids & polymers in comparison with liposomes.

**Major Objective:**

To mask the drug resistance in chemotherapy of NSCLC by silencing drug efflux transporter through siRNA delivery.

It is hypothesized that masking drug resistance will result in increased therapeutic index of chemotherapeutic agent through maximization of efficacy of drug, reduction in dose and possibility of prolongation of therapy due to reduced toxicity. This would result in a complete remission of the disease and in a reduced possibility of metastasis, with consequent increased survival rate of patients.

**Supplementary objectives:**

A. Development of folate receptor ligand conjugated non-viral vectors HLPs and compare its performance with liposomes as carrier.

B. siRNA containing both the carriers will be compared for their performance through *in vitro* and *in vivo* evaluation using appropriate cell lines and animal models, respectively, by administering them through IV and pulmonary route.

Cisplatin is given through Intravenous route and the various drawbacks of conventional cisplatin injection include:

Formualting Dry powder inhaler of HLPs Hybrid Lipo-polymeric vesicles of cisplatin have following advantages:

- Nanosizing of the formulation provides passive tumour targeting through Enhanced Permeability and Retention (EPR) effect. The EPR effect is a result of leaky capillaries adjacent to solid tumors and a lack of a lymphatic system for the drainage of drugs back to the systemic circulation.
- LPHNs might improve the therapeutic index of cisplatin, increasing the drug delivery selectively to tumor tissue. Also nanoparticle formulation are better tolerated and produce less toxic adverse effects than free unentrapped cisplatin.
- LPHNs have structural integrity and stability due to polymeric core and biocompatibility and tissue internalization due to lipid coating, thus combining the advantages of liposomes and polymeric nanoparticles into a single formulation.
- Dry Powder Inhaler of LPHNs would achieve higher concentration of drug at the site of action and mitigate the disadvantages of intravenous route.

# 2. Analytical method development

Cisplatin, cisplatinum, or cis-diamminedichloroplatinum (II), is a well-known chemotherapeutic drug used for treatment of numerous human cancers including bladder, head and neck, lung, ovarian, and testicular cancers. Cisplatin is used in first line treatment of lung cancer. Cytotoxic activity of cisplatin involves both nuclear and cytoplasm component, but its

biochemical and molecular mechanisms of action are still unclear. Its mode of action is linked to the ability of cisplatin to interact with purine bases on the DNA, causing DNA damage, interfering with DNA repair mechanisms and inducing apoptotic cell death in cancer cells. Cisplatin binds with DNA in two steps, first the bond with N7 guanine is formed, and then it binds with guanine or adenine in the same or opposite strand forming inter-strand and intra-strand crosslinks with DNA and causing it's distortion. These Cisplatin-DNA adducts cause the bending and unwinding of the double helix of DNA and loss of function. Cisplatin is administered to cancer patients intravenously as a sterile saline solution. The efficiency of cisplatin administration is often limited by its side effects. Various studies confirmed that cisplatin induces the formation of ROS, esponsible for the numerous side effects like nephrotoxicity , ototoxicity, hepatotoxicity, cardiotoxicity, neurotoxicity alongwith side effects common to most cytotoxic agents such as nausea, vomiting, myelosuppression, gastrotoxicity and some reproductive toxic effects.

Cisplatin (CPT) absorbs ultraviolet radiation at 203 nm, 301 nm and 362 nm but has low molar extinction coefficients of 173, 4.33, 0.806 respectively and has no fluorescence. The low values of extinction coefficients indicate the very less sensitivity of analytical method by UV. Also the method would be hindered by the insolubility of cisplatin in commonly used solvents such as methanol and chloroform. Therefore, a selective derivatizing reaction is required for the detection of the drug in biological fluids if the optical detection is sought. The developed method is based on the ligand exchange reaction of CPT with OPDA.The method is sensitive, precise, reliable, inexpensive, and easy to use. Derivatized Cisplatin absorbs UV-Visible radiation at two wavelengths 430 nm and 705 nm. The absorption maxima of 705 was used for estimation of cisplatin due to significantly higher absorbance at this wavelength. The method was validated for use and interference study was performed.

The experiment was repeated three times. The mean absorbance values with standard deviation were calculated and mean values were plotted against the concentration of cisplatin to get the calibration plot.

**Procedure for Cisplatin Calibration in PBS pH 7.4:**

The stock solution of Cisplatin prepared in PBS pH 7.4 was used and the same procedure described

**Determination of Absorption maxima ( $\lambda_{max}$ ) of Cisplatin**


Cisplatin solutions after derivatization were scanned in the UV-Visible range of 200-800 nm and the absorption maximum was observed to be 705 nm.

**Calibration Curve of Cisplatin in $10^{-4}$ M HCl**

| Concentration (µg/ml) | Absorbance ±SD (n=3) | %RSD |
|---|---|---|
| 1 | 0.146± 0.0035 | 1.6764 |
| 2 | 0.251± 0.0031 | 0.7634 |
| 3 | 0.371±0.0057 | 1.9845 |
| 4 | 0.4901± 0.0042 | 08743 |
| 5 | 0.598± 0.0081 | 1.985 |
| 6 | 0.69912± 0.0068 | 0.873 |

**Conclusion:**

The 1:1 ligand exchange complex formed between cisplatin and OPDA (in 7:3 v/v DMF:Water mixture pH 6.2 was subjected to scanning in UV-Visible Region of 800 nm to 200 nm. The complex was found to absorb at 705 nm. There was no interference between the ingredients of formulation, derivatizing agent and Cisplatin. The Calibration plot was generated at absorption maxima 705 nm and the same was used for further investigating the cisplatin entrapment efficiency in LPHNs. The method was validated for linearity, accuracy, precision, LOD, LOQ. The equation representing the calibration plot was found to be y=0.145x+0.021 and y=0.1987x+0.0276 for Cisplatin derivatized in $10^{-4}$ M HCl and PBS pH 7.4 respectively.

## 3. Cisplatin-caprylate complex formation

Complexation of drugs with polymers has been used for various purposes like increasing drug entrapment in a carrier sustaining drug release and reducing the toxicity, Cisplatin's aquated species are reported to form coordination complexes with various ligands like carboxylic acids, negatively charged phospholipids, and other polymers. In addition, fatty acids can incorporate themselves into the lipid bilayer; such complexation of drugs with fatty acids may increase liposomal entrapment of drugs. Moreover, conjugation with caprylate has been reported to form a lipophilic derivative of tumor necrosis factor (TNF) and enhance its liposomal entrapment. If the complexation of cisplatin is carried out with such a fatty acid, it would result in the formation of a complex that might facilitate interaction with lipid bilayer and thereby improve the entrapment of cisplatin in HLPs. This would also double the advantage because as a liposomal formulation, it can be modified as a long circulating carrier, and this would facilitate the tumor delivery of cisplatin due to enhanced permeation and retention (EPR) effect.
Cisplatin was complexed with caprylic acid (sodium salt) in the molar ratios of 1:1, 1:2, and 1:3. Sodium caprylate was dissolved in water and cisplatin was added gradually while stirring the solution at 60°C so as to have the maximum solubility of cisplatin. Heating was continued until the yellow color of cisplatin disappeared and white-colored complex formed. The resultant dispersion of complex was allowed to cool to room temperature before the preparation of HLPs.

| Cisplatin:caprylate molar ratio | Complex formation | Remarks |
|---|---|---|
| 1:0.5 | - | Yellow cisplatin precipitates were observed on standing |
| 1:1 | + | Uniform white dispersion of complex was obtained. |
| 1:2 | + | Aggregates of white cisplatin caprylate complex observed |
| 1:3 | + | Aggregates of white cisplatin caprylate complex observed. |

## 4. Preformulation Study

Pre formulation studies are designed to recognize the physiochemical properties of drug as well as excipients that may affect the method of manufacture, formulation design, Pharmacokinetic properties of resulting product. This could provide important information for formulation design or support the need for molecular modification. Every drug has intrinsic chemical and physical properties which has been consider before development of pharmaceutical formulation. This property provides the framework for drugs combination with pharmaceutical ingredients in the fabrication of dosage form. Objective of preformulation study is to develop the elegant, stable, effective and safe dosage form by establishing kinetic rate profile, compatibility with the other ingredients and establish Physico-chemical parameter of new drug substances.

**Organoleptic Characterization**

The sample of Cisplatin was checked visually for organoleptic Characterstics like colour and odour of drug.

Color: Yellowish Orange

Odor: Odorless

State: Crystalline powder

All the observed characteristics confirm with the characteristics found in literature

**Melting Point determ ination**

Melting point was determined by adding small amount of Cisplatin in a capillary tube sealed at one end. The capillary tube was placed in an electrically operated melting point apparatus (VEEGO,Mumbai) in which temperature was gradually increased and the temperature at which the drug melt was recorded. Identification of drug confirmed by comparing obtained value with reported value of melting Point for drug.

Melting Point of any compound determines it's purity . Melting point of Cisplatin Determined by Capillary Method was found to be in the range of 280-290°C which was in the range of reported value ( 270-290°C)

**Authentication of drug by Infrared spectrum (IR)**

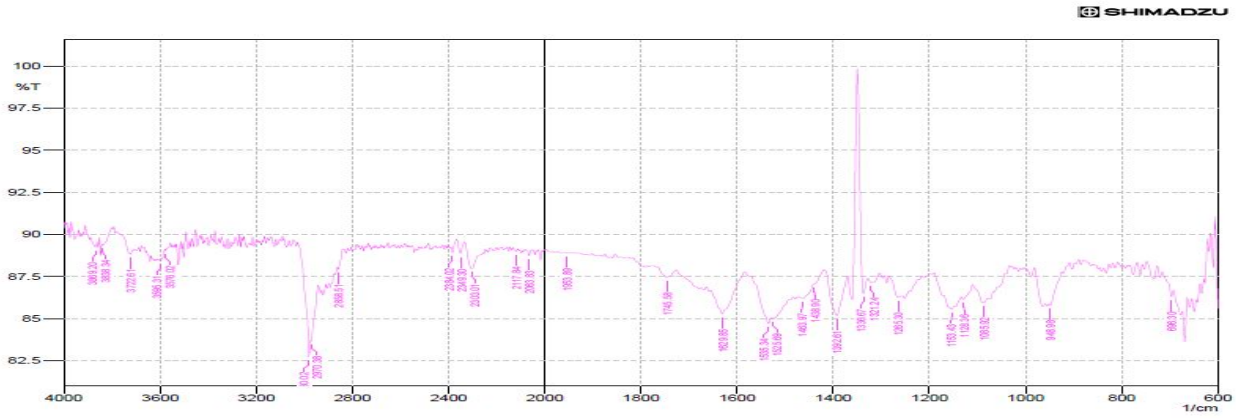
IR spectra were recorded with a IR (Shimadzu IR 8400S spectrometer in range 400–4000 cm-1 using a resolution of 4 cm-1 ) of the Drug and the obtained spectra was matched with the standard spectra obtained in literature

**Drug Excipient Compatibility Study:**

**Infrared Spectrum**

For compatibility Study of Drug and the formulation components , the infrared spectra of Cisplatin and physical mixture of Cisplatin+PLGA+Soy Lecithin was compared. All the components were used in the ratio of 1:1.

The IR spectra of Cisplatin practically obtained was compared with standard IR spectra of drug which was found to show all the characteristic peaks, of functional group of drug. Thus the drug was found to be authentic.




**IR Spectra of Cisplatin**

| Observed frequency ($cm^{-1}$) | Reported frequency ($cm^{-1}$) | Inference | Conclusion |
|---|---|---|---|
| 3010 | 3000-3400 | $NH_3$ Symmetric stretching | All peaks are within the reported range. |
| 1535 | 1525-1540 | $NH_3$ Asymmetric Deformation | |
| 1335 | 1220-1335 | $NH_3$ Symmetric Deformation | |
| 948 | 920-950 | Pt-Cl Stretching | |

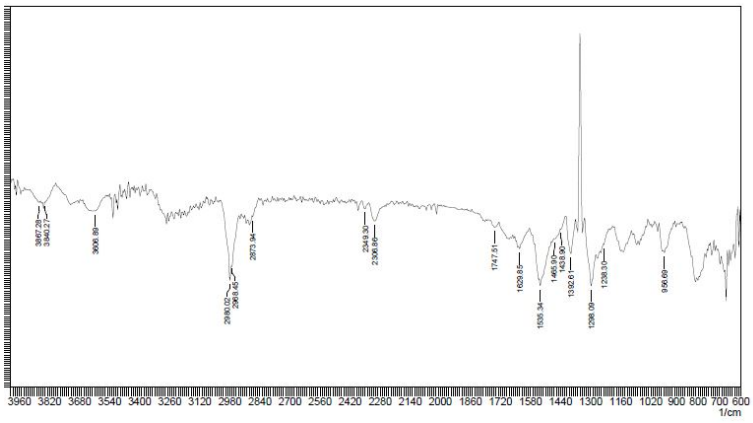
**Interpretation of IR Spectra of Cisplatin**

**Drug Excipient Compatibility study:**

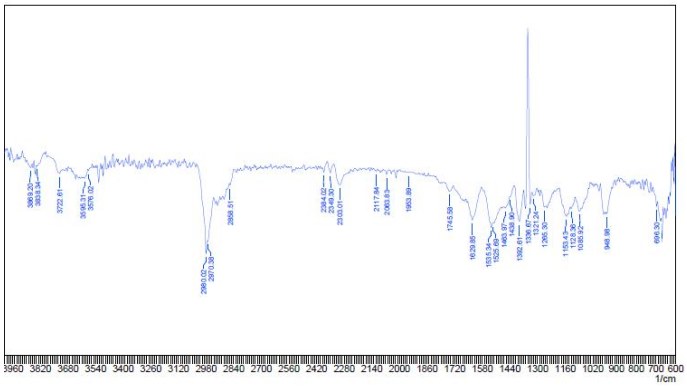
**(A) Infrared Spectrum**

The IR Spectrum of Cisplatin is shown in Fig. The characteristic bands were identified. For the compatibility of drug and excipients, IR of physical mixture of Cisplatin and (Cisplatin+PLGA+ Soy Lecithin) was studied as shown in figure 4.2,4.3,4.4. It exhibited characterstic peaks at 3010 $cm^{-1}$ for $NH_3$ symmetric stretching, 1535 $cm^{-1}$ for $NH_3$ asymmetric deformation, 1335 $cm^{-1}$ for $NH_3$ Symmetric deformation and 948 $cm^{-1}$ for Pt-Cl Stretching Vibrations. All these peaks are considered characteristic to Cisplatin and are prominently observed in IR spectra of Physical mixture as well. No additional peak were observed in physical mixture of drug and excipients.

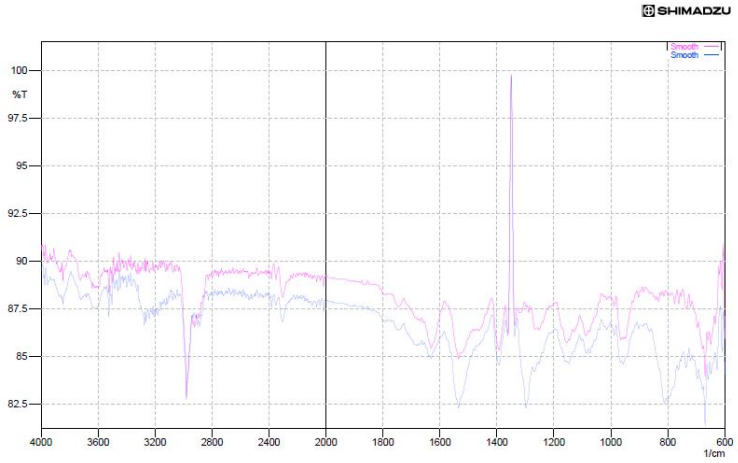
From these results, it was confirmed that there was no interaction between Cisplatin and Physical mixture of Cisplatin and excipients.



IR Spectra of Cisplatin



IR Spectra of Physical Mixture of Cisplatin and excipients



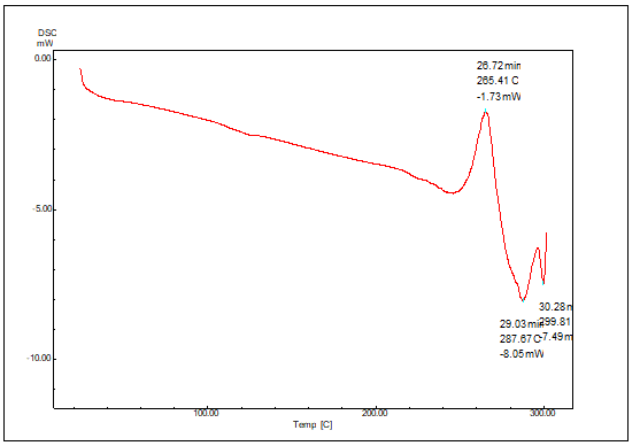

Overlay of IR Spectra of Cisplatin and physical mixture of Cisplatin and excipients

**Differential Scanning Calorimetry(DSC) :**

DSC analysis was carried out using a Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan) at a heating rate of 20°C per minute in the range of 30°C to 120°C under inert nitrogen atmosphere at a flow rate of 40 ml/min. DSC thermograms were recorded for Cisplatin, PLGA Soy Lecithin and physical mixture (Cisplatin+PLGA +Soy Lecithin) in the ratio of 1:1 to check the interference of drug with excipients.

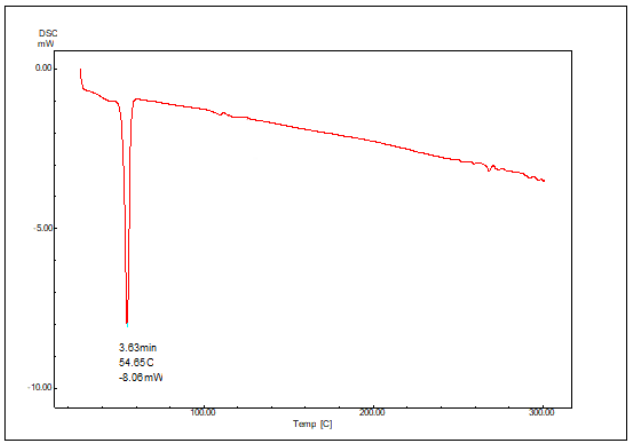
Differential Scanning Calorimetry(DSC) is widely used in thermal analysis to monitor endothermic processes( Melting, solid-solid phase transitions and chemical degradation) as well as exothermic processes ( Crystallization and oxidative decomposition ). It is extremely useful in preformulation studies since it indicates the existence of possible drug-excipient or excipient-excipient interaction in formulation.

Fig shows DSC thermogram of Cisplatin showing sharp endothermic peak at 287 °C corresponding to melting point of Cisplatin. This also confirms the melting point range observed using capillary method. ( 280-290 °C ).



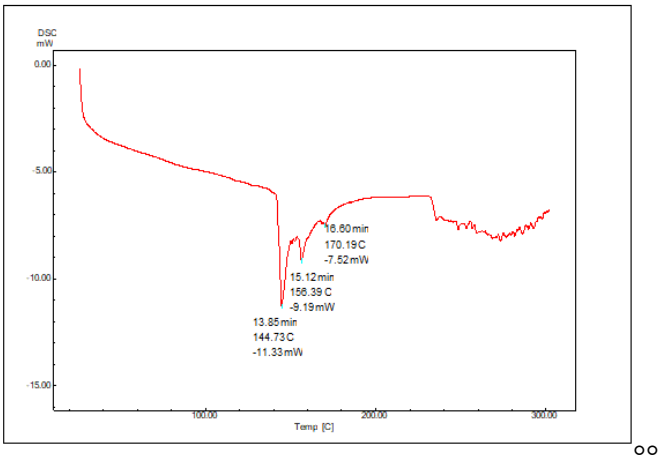

DSC thermogram of Cisplatin

Fig. shows DSC thermogram of PLGA which shows sharp endothermic peak at 54 °C corresponding to the melting point of crystalline PLGA.[5]



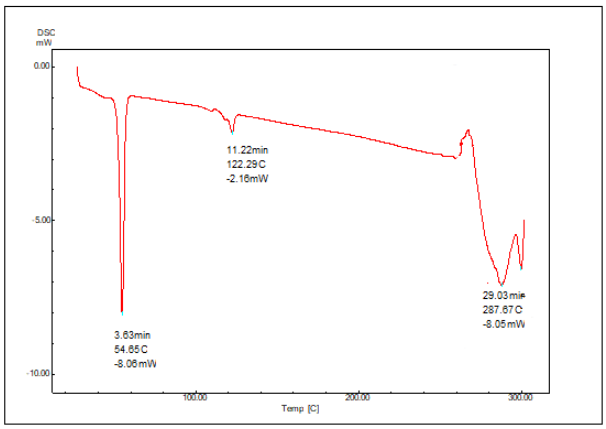

DSC thermogram of PLGA

Fig shows the DSC thermogram of Soy Lecithin and it shows sharp endothermic peaks at 144°C Indicating isotropic liquid state of Soy Lecithin and subsequent peaks at 156-170 °C indicate the boiling point of Lecithin. [6]




DSC thermogram of Soy Lecithin

Figure indicate the DSC Thermogram of Physical Mixture of Cisplatin with PLGA and Soy Lecithin. There was no change in endothermic peak of drug so we can say that the Drug and excipients are compatible with one another.It is known that the quantity of the material used especially in drug-excipient mixtures , could influence the peak shape and enthalpy. Here no change was observed in melting endotherm of drug and mixture of drug with excipients. So drug and excipients are compatible with each other.




Physical mixture of Cisplatin with PLGA and Soy Lecithin

## 5. Formulation development and optimization

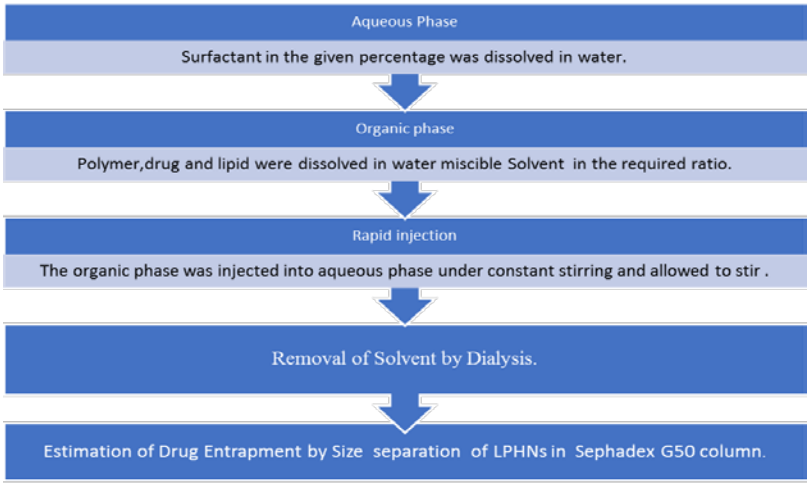
### Introduction

Lipid Polymer hybrid nanoparticles are the amalgamation of properties of liposomes and Nanoparticles providing advantageous size range (10-200 nm) favorable for endocytotic intercellular uptake, accumulation in the tumor site through leaky tumor vascular structures,

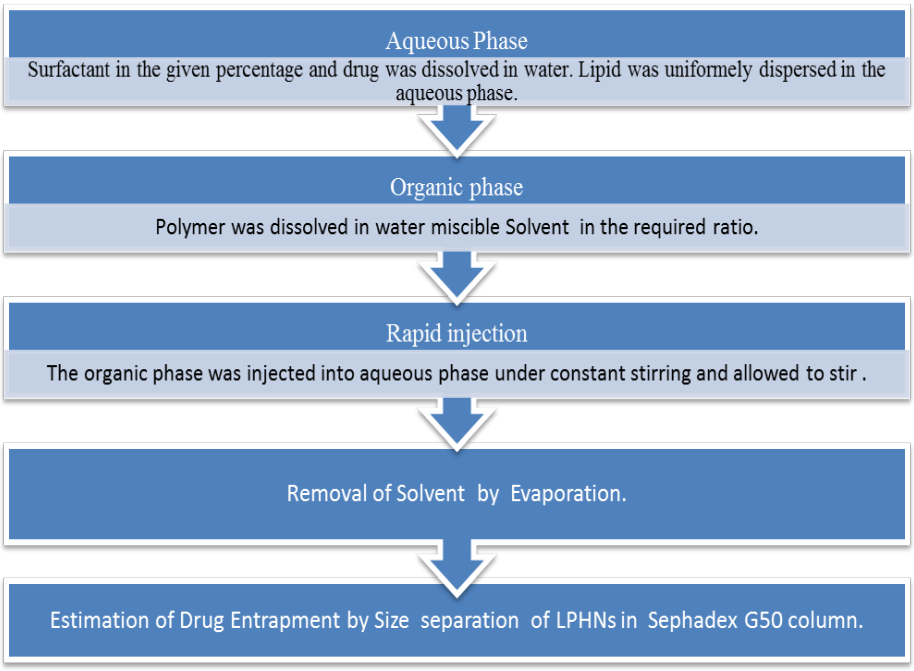
which is useful for prolonged drug exposure to the tumor site and good structural stability. Lipid polymer hybrid nanoparticles can be synthesized through two distinct approaches. One approach involves a two step process in which the polymer core and lipid shell are prepared separately and then merged together; the other approach involves a single-step process, in which the hybrid nanoparticles are prepared through a one-pot nanoprecipitation and self-assembly method. All the methods were checked for their feasibility and the best method suited to prepare Cisplatin LPHN with favourable characterstics was further optimized.

**Single step Nanoprecipitation (Water Miscible Solvent Evaporation )**

This method produced LPHNs with uniform particle size, good entrapment and desired lipid coating and this was further optimized. Figure describes the method in a step wise manner in which Lipid and polymer were both in organic phase. Fig describes the method in which Lipid was in the aqueous phase and polymer in organic phase.




Single Step nanoprecipitation method in which Lipid and polymer were both in organic phase.




Single Step nanoprecipitation method in which Lipid was dispersed in aqueous phase and polymer was dissolved in organic phase.

Formulation Optimization:

| **Material Selection** | **Process Prameters** | **Formulation Parameters** |
|---|---|---|
| Selection of Polymer | Stirring Speed | Lipid:Polymer Ratio |
| Selection of Lipid | Rate of Addition of Organic Phase | Drug:Polymer Ratio |
| Selection of Solvent (Organic phase) | Stirring Time | Surfactant Type |
| | | Surfactant Concentration |

## ***Optimized batch of HLPs containing cisplatin***

| Lipid:polymer ratio | Organic phase: aqueous phase ratio | Surfactant conc | solvent | size | PDI | % Entrapment efficiency |
|---|---|---|---|---|---|---|
| 2:1 Soya lecithin/PLGA | 1:5 | 0.5 % Poloxamer 107 | Acetone | 187.5 nm | 0.121 | 40.71 |
| 1:5 Soy lecithin 1.8 mg DPPG: 0.7 mg( Soy PC:DPPG 7:3 weight basis) DSPE PEG 5 mol% (0.14 mg) (PLGA 12 mg) | 1:5 | 0.5 % Poloxamer 107 | Acetone | 161.5 nm | 0.19 | 31.48 |

**Results and Discussion:**

1) **Assay of LPHNs:**
   The %assay was found to be in the range of 97-100.1%
2) **%Entrapment efficiency:** The %entrapment efficiency was found to be 39.71±1.091 (n=3) in the optimized formulation.
3) **Drug loading**
   The drug loading was found to be 8.1 % w/w(n=3)

4)**Particle size and zeta potential of PLGA nanoparticles:**

To have therapeutic potential as a drug delivery system, drug-loaded LPHNs must possess a narrow size distribution of Sub- micrometer mean, together with a biocompatible zeta potential and an efficient drug loading. In the present study the

Particle size obtained was in the range of 150 to 166 nm (181.76 nm ± 3.91 nm)with very good polydispersity index of 0.192±0.061 .

The Particle Size is an important particle property, as it can influence the biopharmaceutical properties of the particle preparations. The biodistribution of the particles may also depend on the PS. Endocytosis of the particles, is size dependent. The particle uptake is reported to be the size dependent phenomena, where the small sized particles could be efficiently taken up as compared to the bigger sized particles. For Cancer cells, particle size is important to target EPR (Enhanced Permeability and Retention) effect. The zeta potential of PEGylated LPHN was found to be -24 mV ±1.91 mV. Negatively charged Particles will repel one other and provide stability by preventing aggregation

**4) Differential Scanning Microscopy (DSC)**

The DSC thermograms of Cisplatin and Lyophilized Cisplatin LPHNs are shown in figure

The DSC thermogram of Cisplatin showed sharp endothermic peaks at 287°C indicating its melting point. The DSC thermogram of Lyophilized Cisplatin LPHN did not show any Sharp peaks indicating amorphous material. Hence it is evident from DSC studies did that there was no crystalline drug material in the LPHN samples. This shows the crystallinity of the drug has been reduced significantly in the nanoparticles. Hence it could be concluded that in the prepared LPHNs the drug was present in the amorphous phase and may have been homogeneously dispersed in the PLGA matrix.

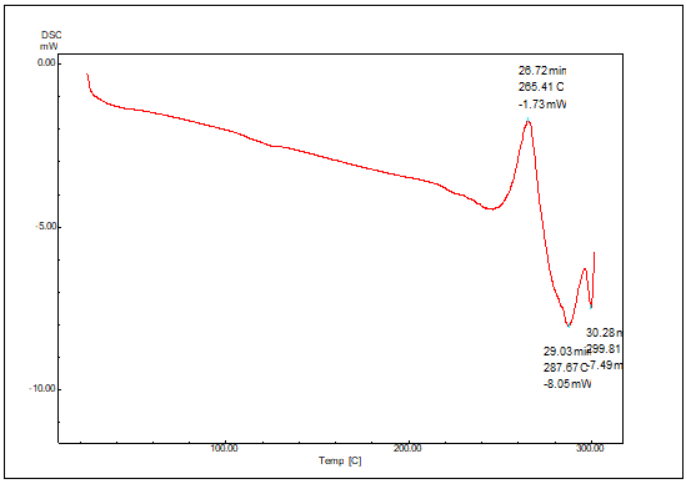
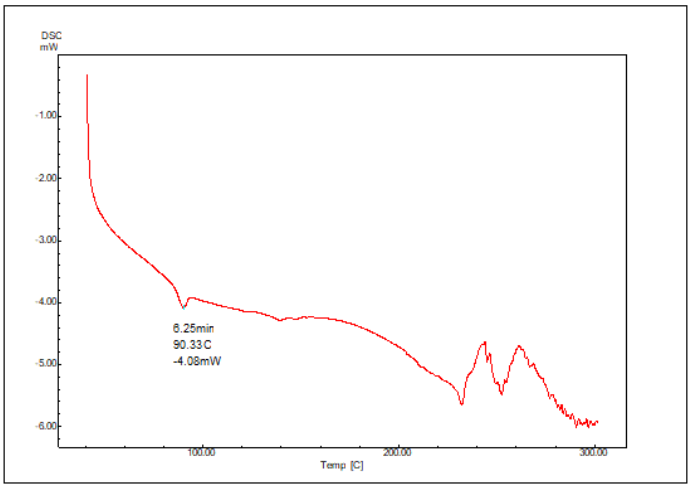


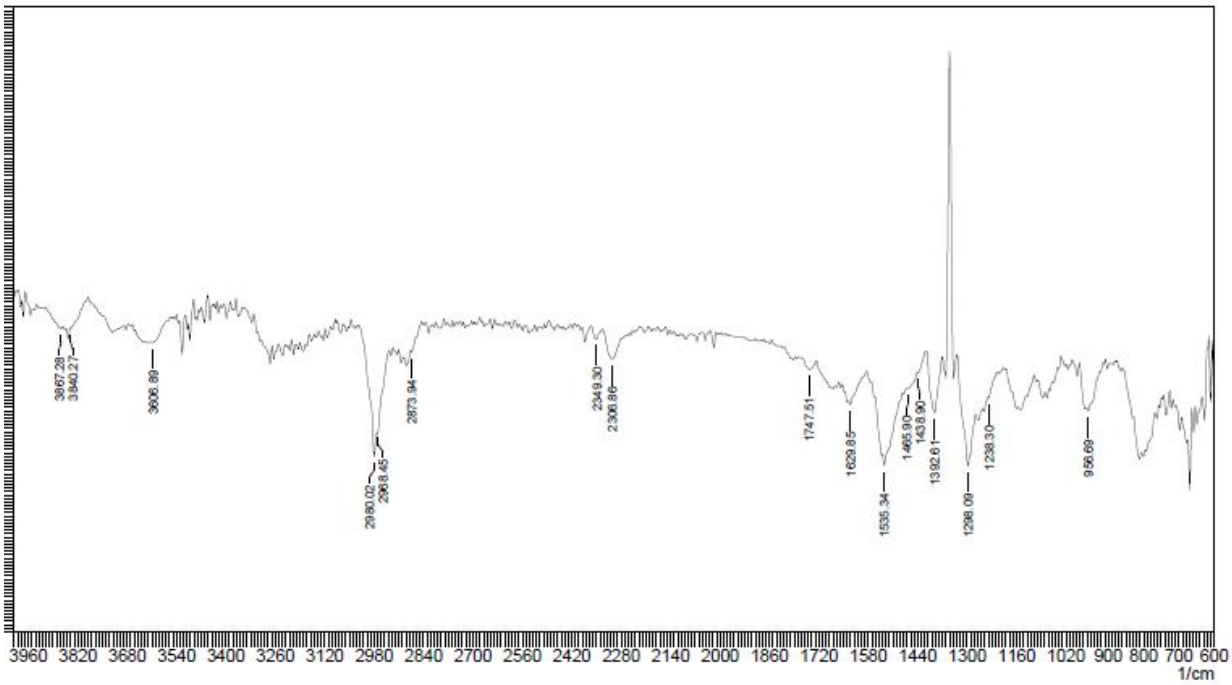

Fig. DSC Of Cisplatin



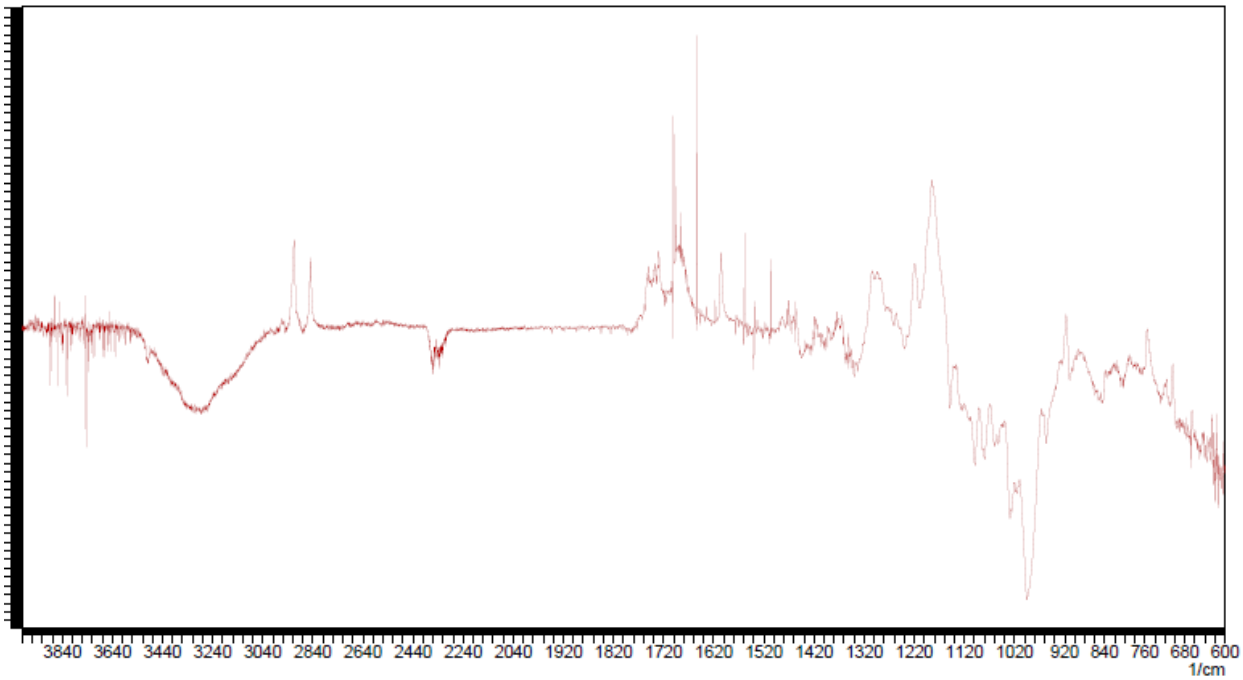

**DSC of Lyophilized Cisplatin LPHNs**

### 5) Infrared Spectrum

The IR spectrum of Cisplatin and Lyophilized Cisplatin loaded LPHN is shown in figure. The spectrum of and Lyophilized Cisplatin loaded LPHN showed retention of the major functional group peaks of Cisplatin(3010 $cm^{-1}$ for $NH_3$ symmetric stretching, 1535 $cm^{-1}$ for $NH_3$ asymmetric deformation, 1335 $cm^{-1}$ for $NH_3$ Symmetric deformation and 948 $cm^{-1}$ for Pt-Cl Stretching Vibrations. ) So it is concluded that any chemical change was not observed in Cisplatin after formulating on LPHN and subsequent lyophilization.



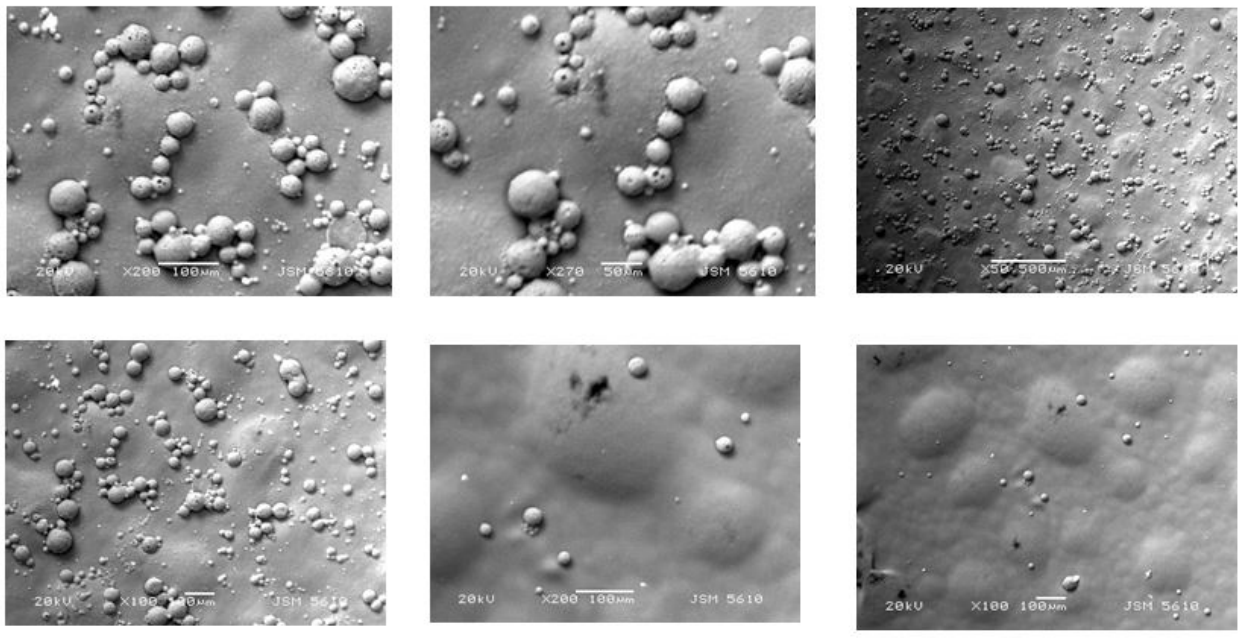

**IR Spectra of Cisplatin**




**IR Spectra of Lyophilized Cisplatin LPHNs**

## 6) SEM Analysis

On SEM analysis of PCl nanoparticles( 600 nm Size) spherical shape nanoparticles were seen thus confirming the feasibility of the method chosen for optimization.



**SEM images of PCl LPHNs**

## 7) TEM analysis

TEM analysis was done for both PLGA nanoparticles and PEGylated LPHNs of Cisplatin. From the comparison of both type of nanoparticles, it can be concluded that the presence of Lipid coating around the PLGA was clearly evident in LPHN formulation. All the particles seemed uniformly coated with average coating thickness of 14-16 nm. The average size of LPHNs from TEM was found to be 141 nm.

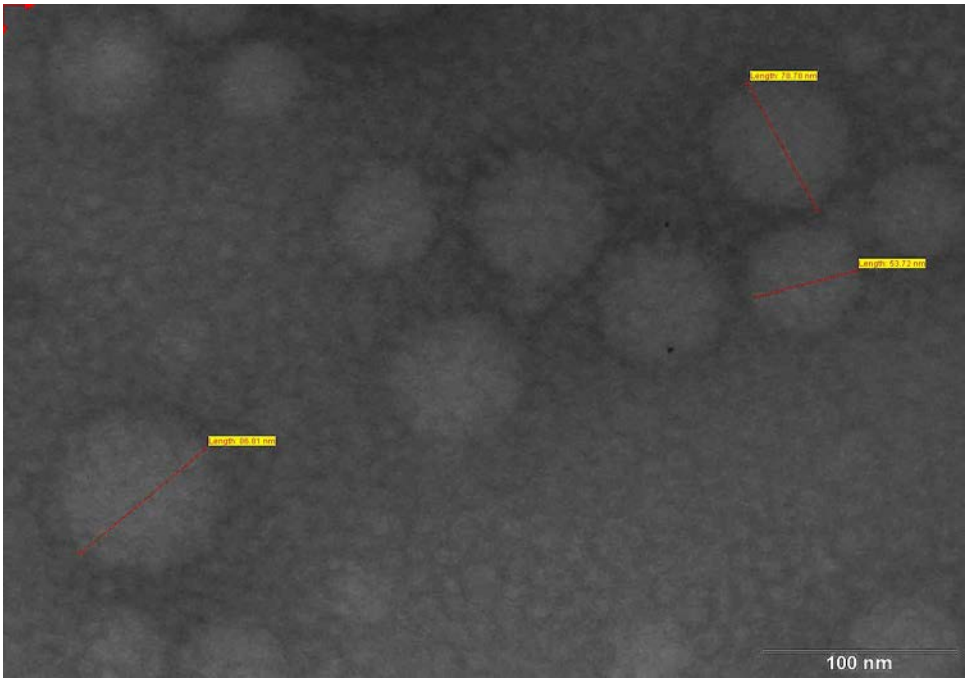
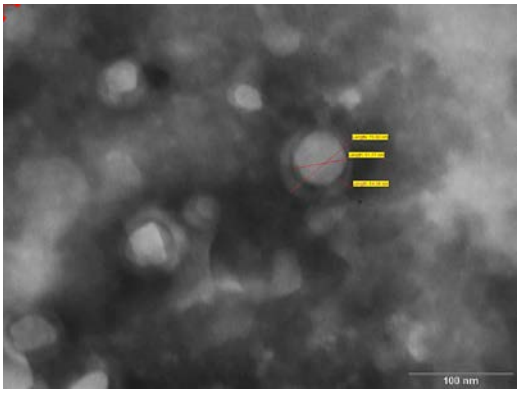



Fig. TEM images of PLGA nanoparticles.



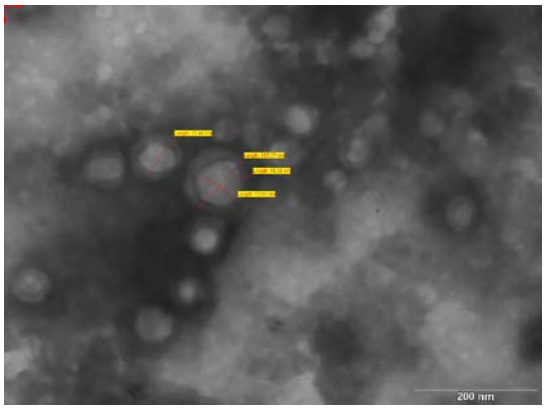



Fig. TEM images of LPHNs

## 8) Electrolyte Flocculation study:

Electrolyte flocculating property of the LPHNs was determined by measuring the absorbance at 400 nm and also the size of the LPHNs and the results obtained were as shown in table below.

**Table: Electro flocculating Property of LPHNs.**

| **Concentration of Sodium Sulphate**<br><br>**% w/v** | **Absorbance** | **Size(nm)** |
|---|---|---|
| **0** | **0.291** | **167.4** |
| **0.5** | **0.313** | **171.1** |
| **1** | **0.323** | **176.5** |
| **1.5** | **0.341** | **184.1** |
| **2** | **0.361** | **187.5** |
| **3** | **1.141** | **324.9** |
| **4** | **1.241** | **351.9** |
| **5** | **1.259** | **353.8** |

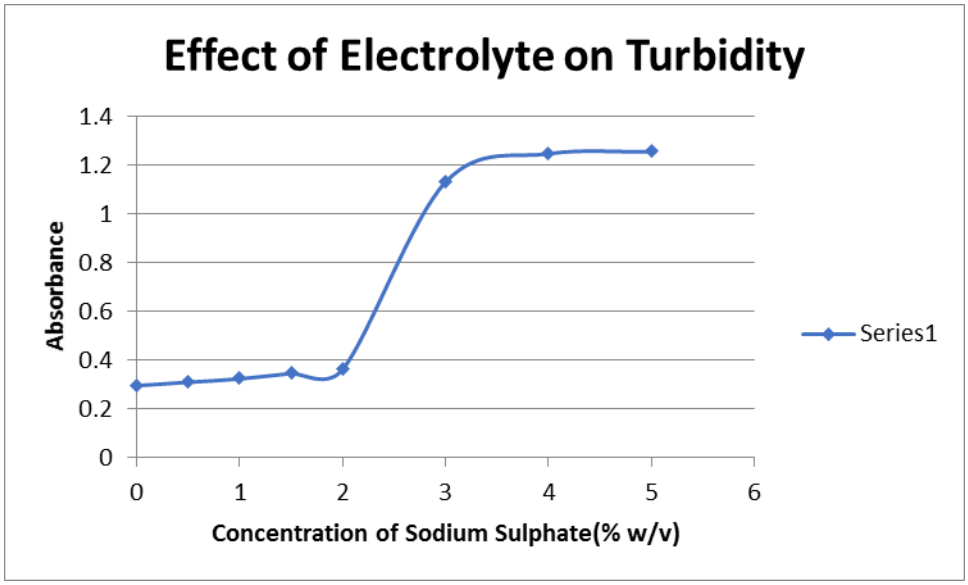



Fig. effect of Electrolyte concentration on turbidity of solution

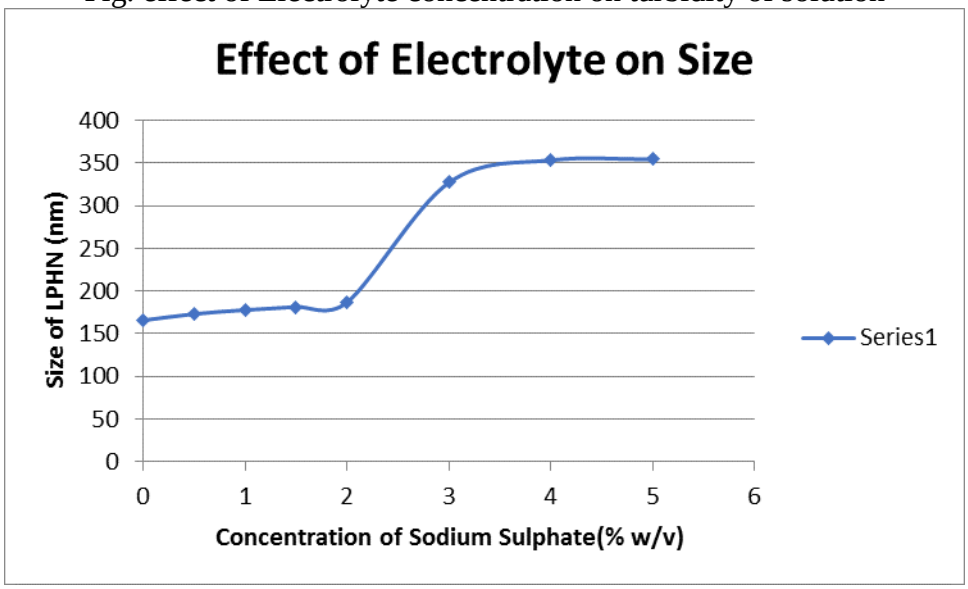



Fig. Effect of Electrolyte concentration on Size of LPHNs

After inhalation, LPHNs would be exposed to various ions including sodium, calcium, potassium, chloride in lung which may affect the stability of LPHNs and also by flocculation, size of the LPHNs also increases, so its clearance will be more from the lung tissue and also increase the macrophage uptake. From this study it was found that LPHN preparation was able to resist aggregation due to external electrolytic influence up to 3 % concentration of $Na_2SO_4$. At higher electrolyte concentration the steric barrier was lost and thus aggregation was found above 3% $Na_2SO_4$ concentration. This was concluded by abrupt increase in the absorbance after 3% $Na_2SO_4$concentration.

## 6. Ongoing work

- Membrane elasticity by micro-pipette aspiration test
- Lipid content in hybrid vesicles
- Quantification of targeting ligand number on vesicle surface
- Fluorescence resonance energy transfer (FRET)
- Atomic force microscopy for detection of lipid coating in HLPs
- Freeze fractured TEM/Cryo-TEM
- Electro-mobility shift assay
- Complexation ability of developed non-viral vectors with siRNA
- Optimized formulation containing cisplatin and siRNA-ABCC3 and its conversion into Dry powder inhaler (DPI) & its characterization study

## 7. Future Plan

- In vitro drug release study
- Cellular uptake study/ In vitro cell line study (before & after nebulization)
- In vitro cytotoxicity study: (MTT assay) (cell lines : FR positive KB/H1299 and FR negative A549 cells adenocarcinomic human alveolar basal epithelial cells)
- Chemo sensitization study
- Transfection study/Gene expression by Real Time PCR
- In vivo study: Safety and Efficacy study

## 8. List of Publications

1. Rohan Lalani, ***Ambikanandan Misra***, Jitendra Amrutiya, Hinal Patel, Priyanka Bhatt and ***Vivek Patel***, Challenges in dermal delivery of therapeutic antimicrobial protein and peptides, Current Drug Metabolism, Volume 18, 2017.(E-pub Abstract Ahead of Print)DOI: 10.2174/1389200218666170222151217

2. Manisha Lalan, Rohan Lalani, ***Vivek Patel and Ambikanandan Misra***; chapter 8; In Vitro and In Vivo Tools in Brain-Targeted Drug Delivery Research, In-vitro and Iv-vivo toold in drug delivery research for optimum clinical outcome; CRC press, Taylor & Francis group, 2017, 237-282.

3. Imran Vhora, Rohan Lalani, Priyanka Bhatt, Sushil Patil, Hinal Patel, ***Vivek Patel and Ambikanandan Misra***; Colloidally stable small unilamellar stearyl amine liposomes for effective BMP-9 gene delivery to stem cells for osteogenic differentiation; AAPS PharmScienceTech (Manuscript accepted)

4. ***Vivek Patel***, Rohan Lalani, Saikat Ghosh, Denish Bardoliwala and ***Ambikanandan Misra;*** Lipid-based oral formulation strategies for lipophilic drugs, AAPS PharmScienceTech (Manuscript under publication)

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