6. Introduction

Among all the liposomal formulations formulated from different amino acids modified DOPE lipid, liposome composed of arginine modified DOPE lipid showed most promising results as confirmed by *in vitro* cell line studies. As a result, liposomal formulation formed from DOPE-A was only selected for further studies.

6.1 Nasal delivery of lipoplex

The nasal course of action presents an immense opportunity to milk the intensively vascularized respiratory airway for systemic drug delivery to grant brisker onset of therapy and diminished degradation of drug equated with conservative oral routes. Computational fluid dynamics (CFD) simulations of nasal drug delivery often forget the preliminary spray particle and as an alternative, commence particles entrained individually by the inhaled airflow facing nasal cavity (1). Hence, data for the validation of spray atomization can be delivered by sub terrestrial measurement and turn particle deposition design of sprayed aerosols in nasal cavity. Spray atomization is an acquainted liquid delivery method in diversified industries, including self-propelling, manufacturing and mineral procedures. Especially in automotive industry, high pressure spray injection is one of the most expansively studied experimentally and numerically procedures. Usually, tablets and injections are the cardinal extend of drug delivery methods. Tablets are delivered through the GI tract of patients. The gastric acid and metabolism in the system has possible jeopardy to motive downfall of physic statement. Drug injection is a method of intravenous therapy management which delivers drug rapidly to circulation. However, the discomfort related to the injection may purpose no-complaisance by enduring. Nasal spray provides additional opportunity which can eschew these hindrances. Drug has been delivered by nasal spray through nasal valve and deposits droplets of drugs onto wall of nasal cavity in turbinated nasal area. Therefore, drug can be expeditiously diffused to the mucosa and widespread blood vessel web found in nasal cavity. Besides, it additionally has potential of amending therapeutic efficacy in systemic health dilemmas, such as cancer, CNS disorders, lung disease and sinus infections (2).

The delivery of spray droplets is proximately related to the structure and design of nasal spray bottle (3). The drug deposition in turbinate nasal will be diminished by the deprived penetration of spray droplets. Hence, improved knowledge of atomization process in nasal spray device can amend the design of spray bottle and drug delivery efficiency can be improved. At present, core focus of nasal spray research is on spray droplets deposition in nasal cavity. Latest researches concerning the radiolabelled nasal spray droplet deposition

in the nasal cavity of ten healthy volunteers by means of gamma scintigraphy by Suman et al. has bettered the understanding of affiliation between efficacy and parameters of drug delivery system (droplet size, plume angle, spray pattern etc).

Actuation parameters research is one of the dominant research area in the field of nasal drug delivery (4). The nasal-pump action has paramount effect on dose delivered and distribution of spray droplet size. Researchers have additionally revealed that the miscellaneous hand force will lead to diverse dosage. According to the guidelines drafted by The U.S. Food and Drug Administration (FDA) (2003), it is suggested that pharmaceutical companies should accomplish spray testing by automated actuators to circumvent the dubious variation of actuation force by miscellaneous hand operations. The force controlled arrangement is pneumatically driven and mimic the trained patients' utilization of nasal spray and the droplet size analysis is accompanied by laser-diffraction technique.

6.2 Formulation of nasal spray

Glycerine was mixed with water in beaker I and stirred well for 30 min. At the same time, in another beaker, beaker II, Di sodium EDTA was dissolved in water; phosphate buffers and sodium chloride were added to the solution once the solution became clear and mixed with the solution of beaker I. Finally, benzalkonium chloride was added as preservative and volume was made up with nuclease free water. The optimised formula for the preparation of nasal spray is represented in table 6.1 below.

Table 6. 1 Composition of nasal spray formulation

Ingredients	Role	Quantity in percentage
Sodium Phosphate, monobasic	Buffer component	0.5525 %
Sodium Phosphate, dibasic	Buffer component	0.0975 %
Di Sodium EDTA	Chelating agent	0.03%
Glycerine	Humectant	0.5 %
Sodium Chloride	Osmotic agent	0.8 %
Benzalkonium chloride	Preservative	0.1 %

6.2.1 Role of nasal spray ingredients

Buffers: Concentration of unionised drug available for its absorption or its permeation through nasal mucosa is affected by the nasal secretion, or in our case, complexation stability of career can be affected by nasal secretion (5). Hence, to sustain the pH in-situ, an acceptable formulation buffer capacity is crucial. Examples of buffer used in nasal spray sodium phosphate, Sodium citrate, citric acid.

Chelating agent: Chelating Agent is a type of antioxidant, controls the oxidation of components present in spray which may lead to its degradation and in the process it can reduce its shelf life. It also plays a helping hand for the action of many antimicrobials by disturbing the structure and integrity of microbial cell walls.

Humectant: Nasal mucosa can dry out in certain allergic or disease conditions. Preservatives or antioxidants used in nasal spray may cause nasal irritation when used in excessive quantities.

Sufficient moisture in nasal cavity is necessary to prevent dehydration. Humectants used in nasal spray avoid such circumstances and they do not affect permeation or absorption of formulation dispersed in spray. Common examples include glycerine, sorbitol and mannitol.

Osmotic agent: Osmotic agents are added to the nasal spray to increase the solute level in formulation containing spray and contribute to achieve hypertonicity in nasal spray (6). In short, osmotic effect needs that the formulation be hypertonic with respect to nasal mucosa. Sugars such as glucose, sucrose and fructose can also be used instead of Sodium Chloride.

Preservative: Nasal sprays are aqueous based formulations so they need preservatives to prevent growth of microbes. Parabens, phenyl ethyl alcohol, benzalkonium chloride and benzoyl alcohol are some of the commonly used preservatives in nasal formulations.

6.3 Characterization of nasal spray

6.3.1 Formulation parameters

pH

for the nasal spray in form of solution and suspension, an appropriate acceptance criteria are established and observed pH value of nasal spray should be within that established criteria. pH of the interior portion of nose ranges from 5.17 to 8.13 while the pH of posterior region of nose ranging from 5.20 to 8.0 resulting in an average pH of human nose is around 6.3. Thus by selecting optimal formulation pH; a stable nasal spray can be formulated. Therefore, nasal spray with pH around 6 prevents sneezing that usually results from nasal irritation. For the measurement of pH, the fixed sample of nasal spray was taken in 50 mL beaker and pH meter electrode was dipped in it. Reading was noted after the steady pH was recorded on Labindia pH meter.

Osmolality

Many experiments have proved that drug permeability through nasal mucosa can be improved by hypotonic nasal spray formulation. Nasal products existing in market showed osmolality in the range of 300-700 mOsmol/Kg (7). The instrument was operated as per manufacturer’s guidelines. For the measurements of osmolality, empty sample tube was

placed inside sample well and test was started; result displayed was recorded and shown in table.

Viscosity

High viscosity of nasal dispersion can increase the contact time between nasal spray and mucosa which may increase the chances of permeation (8). The viscosity of nasal spray formulation was measured using Brookfield viscometer. An appropriate spindle was immersed in the test liquid and rotated at different speeds (10 to 100 rpm). The reading was taken after five full spindle rotations. Three replicate measurements per formulations were carried out at temperature of 25 ±2 º C and shown in table.

6.3.2 Actuation parameters

Droplet size distribution (DSD)

Research and development wing of FDA (Food and Drug Administration) has made it clear to submit nasal spray characterization data for new or existing nasal based drug delivery systems.

This characterisation includes in-depth investigation of spray's cross-sectional ellipticity which is commonly known as plume geometry, spray pattern which is important for uniformity of droplet distribution, shot weight and droplet size distribution of nasal spray. Droplet size distribution (DSD), is the determining factor for where the droplets of nasal spray would locate in nasal cavity which ultimately affect the bioavailability of API (drug/cDNA/siRNA) which depends on the spray pump from which it is actuated (9). For instance, slow actuation probably results in poor atomization creating a flow resembling stream. And the same way, fast actuation may generate fine spray which may result in poor absorption from the nasal cavity and droplets may deposit in throat and lungs. The laser diffraction particle analyser offers a faster and precisely evaluate the droplet size distribution within the spray plume (10). For the light source, a laser unit is used; when laser pass through droplet, detector (a receiving unit here) evaluate the diffraction pattern. This diffraction pattern is used to develop the droplet size distribution graph. Various drop size measurement instruments are given below in figure 6.1.

Droplet size distribution was determined by laser diffraction technique employing HELOS BR Instrument with SPRAYER module and force actuator. Various instrument parameters like spraying angle (0, 30, 60, 90), actuation force (35, 45, 55), actuation distance (3, 5, 7 cm) and stroke length (2, 4 and 6 mm) were optimised at 2 sec of hold time. Time resolved measurement was performed and data were analysed by Fraunhofer theory (11).

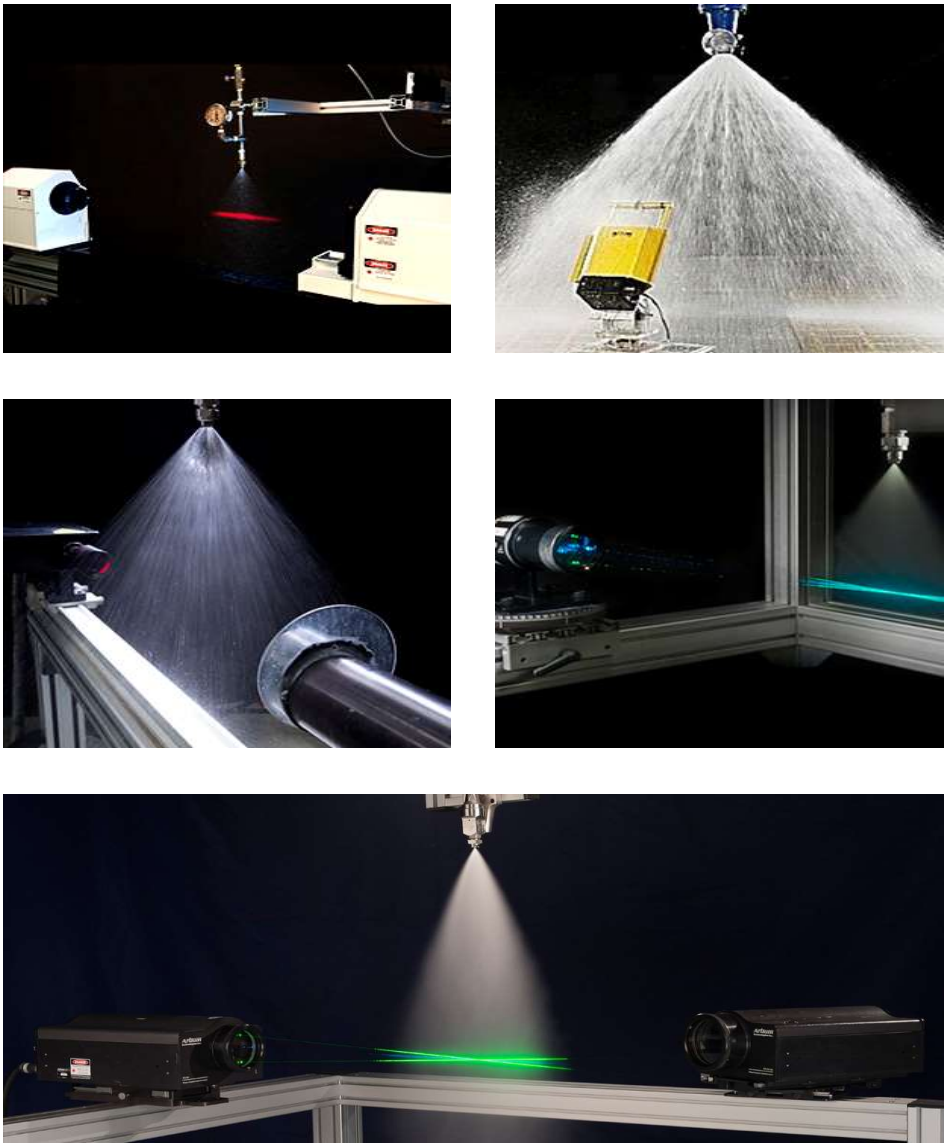

Figure 6. 1 Droplet size measurement instruments

Plume geometry and spray pattern

During early stages, these evaluations usually used to assess spray performance to result in cost effective and more efficient product. However, no pinpoint method is available that predicts the spray performance is not in existent. Measurements of plume geometry and spray patterns are main criteria for the consistent results for nasal spray performance. Plume geometry is defined as a side view of nasal spray cloud parallel to plume axis. Laser light and high-speed photography are appropriate methods to assess plume geometry.

As stated earlier, instruments like sprayVIEW NSP system and sprayVIEW software are designed specially with high speed camera and laser light source, were used to analyse plume geometry and spray pattern of nasal spray (12). As per USFDA guidelines, these instruments were precalibrated for actuation time and force for the spray pump filled with the sample to be tested was kept in actuator mouth. Not only droplet size, plume geometry and spray pattern also contribute in predicting droplet deposition in nasal cavity. Nasal spray with wide plume angle tends to deposit in anterior region of nasal cavity while narrow angled nasal spray prominently lean towards turbinate region (13).

Shot weight

Shot weight was assessed by weighing the spray pump filled with 10 gm nasal spray prior to and after each actuation using an analytical balance with maximum weighing capacity of 200 gm and sensitivity of 0.1 mg.

6.4 Ex- vivo characterisation

6.4.1 Preparation of Nasal Mucosa Membranes

Nasal mucosa from goat nostrils were isolated very carefully. The snouts were detached from the animals and conchae was exposed. Mucosa from the nasal cavity was removed using forceps and scalpel, resulting in two sections in each snout. Chloroform-methanol in 2: 1 volume ratio was used to remove lipids from mucosa (14). This chloroform-methanol mixture extract all the lipids from mucosa so no UV absorbance due to lipids was observed and stored at -80 ℃ for future use. Before actual using this delipidised mucosa for carrying out permeation studies, it was primarily hydrated for a day in isotonic phosphate buffer at pH 7.4 on ice bath.

6.4.2 *Ex vivo* Permeation Studies

Ex vivo permeation studies were carried out using a diffusion cell of vertical type (15). Sheep mucosa was mounted on this chamber with nasal mucosa facing the donor compartment and serosa mucosa facing the receiver compartment. The receiver compartment was filled with freshly prepared PBS (pH 7.4) maintained at 37 ± 2 º C with continuous stirring in a way that serosa mucosa just touches the buffer filled in. Subsequently, desired concentration of suitably diluted lipoplex dispersion was added to the donor compartment. 0.5 ml sample was withdrawn from the receptor compartment at predefined time intervals and fresh PBS (pH 7.4) was added to receptor compartment. The withdrawn samples were suitably diluted and analysed by fluorimetry.

6.4.3 *Ex vivo* nasal toxicity study

Toxicity is a main problem in developing formulation for any route including nasal delivery (16). Changes in the histopathological levels, is a sign of toxicity because of variety of ingredients incorporated in the formulation. As an entrance to the brain, nasal cavity is the crucial site of injury concerned with natural lipids and synthesised lipids, moreover, ingredients used in the preparation of stable nasal spray. All these components, may have advert effects on the integrity of nasal epithelial cells or they may affect the mucociliary clearance. As a result, studying the effects of these components on nasal cavity, in an *ex vivo* model is a major aspect of preclinical studies involving nasal formulations.

6.5 Systemic delivery of antibody-directed liposomes

Immunoliposomes (antibody-directed liposomes) are one of the most promising tool for targeted or site specific delivery of API and diagnostic agents alone or together. But its *in vivo* application is hindered by is rapid clearance form body by RES system (17). This hindrance can be avoided by entering PEG conjugated phospholipid as one of the component in liposome formulation, as this modification increases the circulation time of liposome within body. Such liposomes formulated from biocompatible PEG conjugated lipids are commonly referred to as “stealth liposomes”. Moreover, this PEG chains provide the sterical hindrance to liposomes, they are also referred to as “sterically stabilized” liposomes (18). Coating of PEG prevent plasma protein binding to liposomes resulting in non-recognition liposomal vesicles (stealth) for macrophages. Unluckily, it is tough to associate steric stabilization of liposomes with mAb targeting (immunotargeting). PEG coating may result in steric hindrances for mAb to act together with formulated liposomes. Therefore it is recommended to attach mAb ligand to the distal end of a lipid which is conjugated to PEG chain rather than associating ligand to PEG coated liposome. Non-targeted liposomes are not able to penetrate BBB to deliver its cargo in CNS *in vivo* as these conventional formulations can not pass through tight epithelial junctions of endothelial wall rather they can be used for systemic delivery. But receptor located on BBB specific monoclonal antibodies (mAbs), undergo receptor mediated transcytosis through BBB. The mAb used in these studies is the IGF2 mAb to the IGF receptor, which is abundant on brain microvascular endothelium (19).

IGF-II mAb

Very specific delivery of liposomes across BBB through conjugating monoclonal antibodies increase the diffusion of liposome through brain in comparison to conventional drug delivery system. By this mean, the confidence for CNS therapeutics to cross BBB, have

been kept alive and improved CNS efficacy achieved by above mentioned approach. This concept was actually given in early 90s, as Trojan Horse Concept. Certain BBB receptors, including transferrin receptor (TfR), insulin receptor and LRP-1, transport their ligands into the brain by continuous transporting between luminal and abluminal (brain-facing) membranes of brain endothelial cells, a development identified as receptor-mediated transcytosis (RMT) (20). Antibodies/peptides that bind to these receptors have been designed as molecular Trojan horse to transport genetically or chemically related therapeutics via above mentioned shuttle system.

An Igf mAb can be used to target therapeutics in systemic delivery from blood stream to brain cells to the other side of BBB and preferred molecule is IGF-II mAb. Insulin-like growth factors II is an imperative regulator for signalling that facilitates cell growth and metabolism. These receptors are extensively expressed during all phases of life.

SDS-PAGE

When electrophoresis gel matrix is used to isolate protein of interest, proteins small in size are less resisted due to gel matrix. Other than size of the proteins, the structure and charge on proteins also influence their migration during electrophoresis process. This difference in size and charge can be eliminated by SDS (sodium dodecyl sulfate) as shown in figure 6.2 and gel matrix composed of polyacrylamide. These two parameters affect proteins in a way that they are separated only on the basis of their chain length. SDS is a non-ionic surfactant or detergent that binds to backbone of proteins at a fixed molar ratio which has a strong protein denaturation capacity. For the proper folding of proteins, the presence of SDS along with a reducing agent that cleaves disulphide bonds is very crucial.

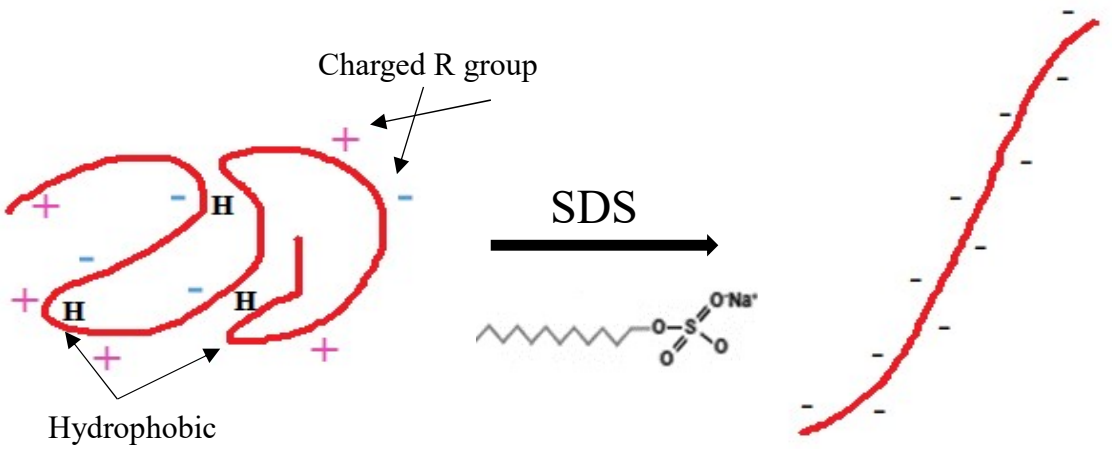

Figure 6. 2 SDS mechanism of action

There are two types of electrophoresis system according to their polyacrylamide gel concentration: “continuous system” and “discontinuous system”. The biggest plus point in “discontinuous system” is their greatly improved isolation capacity or in other words their resolution. In the experiments, gel matrix is divided in two portions: stacking gel and separating gel. Stacking gel is the upper layer of gel matrix and composed of macroporous gel at lower concentration with buffer system of Tris-HCl, pH6.7. In the same way, separating gel is lower portion of gel matrix composed of whole glue at very high concentration with buffer system of Tris-HCl, pH 8.9. Tris-glycerine at pH 8.3 was used as a buffer system in electrophoresis tank.

Evidently, concentration of gel, its composition and pH, the electrophoresis buffer system are not same in this system thus forming a “discontinuous system”. In this system, on applying power; Glycine, proteins, chloride ions and bromophenol in HCI would form negatively charged ions that form flow of ions which move for the anode. As describe earlier, mobility is governed by the net charge and molecular size.

In stacking gel the movement of different ions are in order of glycine <protein <BPB <Cl because on entering the stacking gel from electrophoresis buffer of pH 8.3, glycine ions face the lower pH of 6.7 i.e. almost the isoelectric point (5.97) of glycine, the dissociation of them drop down and their charge would decreased significantly and their movement decrease. The same effect works on protein entering the stacking gel, but the degree of impact is very lower than that of glycine mobility. Moreover, the larger pore size of stacking gel is too big to obstruct molecules of proteins.

As glycine enters the stacking gel, its lower dissociation degree results in rapid lack of mobile ions flow which results in decreased conductivity and subsequently, electric current

also decreases slowly (21). But current in other portion of system continued to be same as before. In this system, there found to be a potential gradient between chloride ions and slow glycine ions which results in conductance. Proteins in this region of gradient difference, move towards chloride ions at variable speeds depending on their size under the effects of conductance. According to this process, proteins accumulated at same place and organized themselves in a way to custom a layer. Proteins bump into resistance from 8.9 pH buffer when they move in to the section of resolving gel and their movement became slow. Meanwhile at 8.9 pH, glycine would dissociate completely. Their net charge would increase and also their electricity, abolishing the chances of missing ion. Now proteins start to move at varied rates owing to the small pore size which provide the gel its sieving properties. Because of this sieving property of resolving gel, small protein-SDS complex moves faster than their counterpart large protein-SDS complex.

As discussed in principle above, main advantage of this type of system is that when they pass through stacking gel section, they create a tight layer and then move into separating gel. As the protein layers separated well before entering into stacking gel as a compressed layer which helps them in diminish the intervention from the overlapping zone, meanwhile also expands the capacity of the system to isolate proteins. Because of this reason, a very small amount of protein samples can also be isolated from this system.

Staining Protein Gels with Coomassie Blue

Positive amine group (basic) of proteins interact with acidic group of dye (sulfonic acid groups of Coomassie dyes R-250 and G-250) through Van der Waals forces (22). Coomassie R-250 is prominently used dye of two and can identify even 0.1 ug of protein. While G-250 type of coomassie dyes used for rapid detection of protein in larger quantity. This is because, staining with Coomassie G-250 does not require any destaining step. Coomassie G-250 establishes a leuco form in very acidic condition below pH 2 (23). Coomassie G-250 dye solution, is dark blue at neutral pH and turn into a clear solution in acidic condition around 2 pH. Leuco form present in this dye regain its original blue colour upon binding to protein as they have more neutral pH in their surroundings. Under favourable conditions, gel placed in acidic solution of this dye, establish blue protein bands within amber background gel. Because of this reason, destaining stage is not require with this dye. Though the protein bands formed from this G-250 dye is less sensitive than R-250 dyes.

6.6 Functionalisation of liposomes

Functionalisation of liposomes were carried out according to pre-insertion and post-insertion method. For pre insertion method, DSPE-mPEG from optimised liposomal formulation was replaced with DSPE-mPEG$_{2000}$-meleimide and rest of the procedure remain same as that of the formulation of liposome.

For post insertion method, 1 mole % of DSPE-mPEG$_{2000}$-meleimide was used to prepare micellar solution and pre-equilibrated at 55 ℃ in RBF (24). Optimised batch of liposomal dispersion was kept at a temperature above the Tg of highest melting lipid (55 ± 2 ℃) for 10 min. The above prepared pre-equilibrated micellar solution of linker lipid was dropwise added to the liposomal dispersion. The mixture was incubated at 55 ℃ and 100 RPM for 3 hrs. After incubation period, the functionalised liposomal formulation was passed through sepharose CL-4B column to separate the micelles from liposomal fraction and analysed for size and zeta potential.

6.6.1 Quantification of sulfhydryl groups

Ellman's assay was used for confirmation of DSPE-mPEG$_{2000}$-meleimide functional moiety to liposomes, after both pre-insertion and post-insertion method of introducing meleimide functional group to liposomes (25). A known amount of thiol is reacted with meleimide moiety and unreacted amount of thiol is determined using ellman's reagent. Thus, this method is an indirect method for the estimation of maleimide. Here, the known amount of thiol was provided by cysteine, which reacts with maleimide functional moiety. The excess of cysteine remaining after reaction with meleimide was quantified by ellman's assay.

Table 6. 2 Test tube contents for quantification of sulfhydryl group

Test tube	Contents
Reaction blank	Non-functionalised liposomes + sodium phosphate containing 1 mM EDTA
Control	Non-functionalised liposomes + cysteine HCl (1.5 mM) + sodium phosphate (pH 8, 0.1 M) containing 1 mM EDTA
Sample 1	Functionalised liposomes by pre-insertion method + cysteine HCl (1.5 mM) + sodium phosphate (pH 8, 0.1 M) containing 1 mM EDTA
Sample 2	Functionalised liposomes by post-insertion method + cysteine HCl (1.5 mM) + sodium phosphate (pH 8, 0.1 M) containing 1 mM EDTA

Ellman's assay was used for the quantification of sulfhydryl group present on the liposomes in form of maleimide functionalisation.

A. Material Preparation

- Reaction Buffer: 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA
- Ellman's Reagent Solution: Dissolve 4 mg Ellman's Reagent in 1mL of Reaction Buffer.

B. Measurement of Absorbance

- 50 µL of Ellman's Reagent and 2.5 mL of reaction buffer was used for each unknown sample to be analysed as shown in table 6.2.
- 250 µL of sample to be tested was taken first step. For the blank sample, instead of taking sample, same quantity of reaction buffer was taken in first step.
- It was mixed and incubated for 20-30 minutes.
- Blank reading was taken at 412 nm (lambda max) and then absorbance for all the samples to be analysed was taken at lambda max after setting up auto zero.
- Amount of sulfhydryls groups present in sample to be analysed was calculated from its molar extinction value of 14,150M^{-1} cm^{-1}

6.7 Preparation of immunoliposomes

Monoclonal antibody was thiolated before conjugated to functionalised liposomes. In thiolation, Sulfhydryl group was introduced through easy surface modifications by Traut's reagent. 0.15 M Na-borate buffer/0.1 mM EDTA (pH 8.5) was used to solubilise mAb which was followed by addition of Traut's reagent (26). The mixture was incubated at room temperature for almost an h. Centriprep-30 concentrator (Amicon) containing 0.1 M Na-phosphate (pH 8.0, 0.1 M) buffer was used to concentrate mAb solutions. Subsequently, thiolated mAb was used to conjugate it with liposomes and evaluated for its size, zeta potential and complexation efficiency.

After thiolation, Monoclonal antibody was incubated with maleimide functionalised liposomes formulated from DOPE-A lipid as a cationic charge providing moiety, in weight ratio of 1: 50. The incubation was kept overnight in cool condition under inert atmosphere of nitrogen (27). Then after, formed immunoliposomes were incubated in same condtion with excess amount of cysteine so that leftover (unconjugated) maleimide groups were occupied by cysteine. The extra unreacted cysteine was removed using ultracel membrane (50 KDa) and was characterised for size, zeta potential, complexation efficiency and

permeation ability. mAb conjugation to liposomes were confirmed by bradford's method SDS-PAGE using coomassie staining (28).

6.7.1 Characterization of immunoliposomes (IL)

Size, zeta potential and N/P ratio

The prepared ILs were characterised for its size, zeta potential N/P ratio for maximum complexation efficiencies as reported in earlier chapter.

SDS-PAGE analysis

The non-reducing, SDS-PAGE (sodium dodyl sulphate-polyacrylamide gel electrophoresis) technique was performed to evaluate and confirm mAb conjugation to formulated liposomes. The detailed procedure of SDS-PAGE is given as below:

The SDS PAGE gel in a single electrophoresis run can be divided into **stacking gel** and separating gel. Stacking gel (acrylamide 5%) is poured on top of the separating gel (after solidification) and a gel comb is inserted in the stacking gel. The percentage of acrylamide in SDS PAGE gel rest on the size of the protein to be isolated from the sample as shown in table..

Table 6. 3 Acrylamide percentage required for the separation of proteins

Acrylamide %	M.W. Range
7%	50 kDa - 500 kDa
10%	20 kDa - 300 kDa
12%	10 kDa - 200 kDa
15%	3 kDa - 100 kDa

30 % w/v Acrylamide solution

It was prepared by addition of 29 gm acrylamide (29 % w/v) and 1 gm of bis-acrylamide (1 % w/v) in 100 ml of miliq water. The pH of prepared solution was confirmed to be 7. The solution was stored in dark at 2-8 ℃ in amber coloured bottle.

1.5 M Tris solution (pH 8.8)

18.16 gm of tris base was dissolved in 100 ml of milliQ water to get a 1.5 M Tris solution. pH was checked and it was adjusted by HCl to around 8.8.

1 M Tris solution (pH 6.8)

9.08 gm of Tris base was solubilised in 50 ml of milliQ water to get a 1.0 M Tris solution. pH was determined and it was adjusted by HCl to around 6.8.

20 % SDS solution

20 gm of SDS was added in 100 ml of miliQ water and heated up to 70 ℃ to solubilised SDS completely. The volume was adjusted to 100 ml after heating.

10 % w/v ammonium persulphate (APS) solution

100 mg of APS was weighed and dissolved in 1 ml of milliQ water. In each SDS-PAGE experiments, it was used freshly prepared.

Tris-Glycine Electrophoresis Buffer (Tank buffer)-5X

It was prepared by addition of 250 mM Glycine, 25 mm Tris base and 0.1 % SDS in milliQ water. The 5X buffer was stored at 2-8 ℃ and the working 1X buffer was prepared by diluting

the 5X buffer with milliQ water to 1X.

Sample loading buffer

it was prepared by addition of 30 mg of Tris-cl, 100 mg of SDS, 5 mg of Bromo Phenol Blue and 0.5 ml of glycerol in milliQ water and the volume was made up to 5 ml.

1X loading buffer

50 mM tris-Cl (pH 6.8), 2% w/v SDS, 0.1 % bromophenol blue, 10 % v/v glycerol—composition of resolving gel and stacking gel are provided in table.

Table 6. 4 Resolving gel and stacking gel compositions

Final Resolving Gel Percentage	
Stock Solutions	Volume in mL
1M Tris pH 8.8	3.75
20% SDS	0.05
40% Acrylamide	2.50
H_2O	3.73
10% Ammonium persulfate	100 µL
TEMED	10 µL
Stacking Gel Recipe	
Stock Solutions	Vol in mL
1M Tris pH 6.8	0.63
20% SDS	0.05
40% Acrylamide	0.83
H_2O	3.4
10% Ammonium persulfate	50 µL
TEMED	5 µL

Coomassie Blue staining method

1. Fix gel in Fixing solution for 1 hr to overnight with gentle agitation. Change solution once at first 1 hr.
2. Stain gel in Staining solution for 20 min with gentle agitation.
3. Destain gel in Destaining solution. Replenish the solution several times until background of the gel is fully destained.
4. Store the destained gel in Storage solution.

All the solutions used in this method are given in table below with their components.

Table 6. 5 Compositions and conditions for staining and destaining solutions

Solution	Components
Fixing solution	50% methanol and 10% glacial acetic acid
Staining solution	0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid
Destaining solution	40% methanol and 10% glacial acetic acid
Storage solution	5% glacial acetic acid)

6.7.2 Bradford's method for estimation of mAb conjugation

To measure the amount of protein present in sample, Bradford protein assay was used (29). This technique was invented by Bradford. It works on a simple principle that the binding of coomassie dye to protein in sample, under acidic pH results in colour change from brown to blue. Moreover, it is a type of colorimetric assay, so as per principle, the darker colour in test sample indicates higher protein concentration present in sample at 595 nm for coomassie dye. Concentration of protein is assessed by comparing it to a series of standard protein solution that shows a linear absorption at 595 nm. For this standard protein solution, from the wide variety of choice, we chose here BSA (Bovine Serum Albumin) as our standard. This method actually measures the alkaline amino acid residues like arginine, histidine and lysine that forms a stable complex with dye. However, the presence of smaller amount of SDS can hinder the protein-dye complex formation and the existence of reducing agents like DTT and metal chelators may hamper the reproducibility of results.

For the Bradford's assay, to the 0.5 ml of immunoliposomal fraction, 2.5 ml of methanol was added to dissolve all lipids. Methanol was then evaporated at 50 º C to precipitate all the lipids. These precipitated lipids were than separated by centrifugation and the supernatant containing mAb was estimated by Bradford's method. For this assay, 10 µl of extracted mAb solutions from supernatant were added to 96 well plate. To each well 100 µl

of Bradford's reagent was added and absorbance was taken at 595 nm using ELISA plate reader.

6.8 Cell cytotoxicity and Cellular uptake study of IL

Cell cytotoxicity and Cellular uptake studies for targeted lipoplex formulation was carried out as per protocol depicted in chapter of cell line studies. mAb conjugated DOPE-A liposomal formulation was compared to its counterpart, non-targeted liposomal formulation to check for the effects of antibody conjugation on cell cytotoxicity and cell uptake. Live imaging was performed using confocal microscopy to access the potential of mAb conjugated liposomes on cell uptake. It was carried out on SHSY5Y cells using naked cDNA, targeted and non-targeted lipoplex containing modified DOPE-A. Protocol of cellular uptake from *in vitro* cell line chapter was followed up to treatment step. Soon after transfection imaging was started.

6.9 *In vivo* experiments

After optimizing DOPE-A formulation for nasal and systemic delivery, they were further assessed in animals for their toxicity and efficacy. All experiments and protocol described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda and with permission from committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

6.10 Intravenous acute toxicity studies

Acute toxicity is adverse biological effect or effects, which occurred within a short period of time after a short-term exposure (30). Many information can be obtained from a well-designed acute toxicity studies including LD50 value of formulations which is a vital information.

Acute toxicity studies provide following information other than LD50 value:

- Any chances of acute toxicity in humans;
- Evaluation of safe dose devoid of any toxicity in humans;
- Possible targets for organ toxicity;
- Tine span for chemically tempted clinical observations;
- Suitable dose for multiple dose induced toxic effects;
- Species variables for toxic effects.

Principally, information related to acute toxicity has been acquired from a unit dose studies in minimum two different types of species using both, to be marketed dosage form and intravenous route (31). This type of information can be acquired from suitably and well

directed dose escalation studies. Only lethality should not be proposed as an endpoint for acute toxicity studies. In this type of study, usually individual animal receives a unit dose of the formulation.

Sometimes, multiple doses may also be administered but should be given within time period of a day or less than a day and the main aim of the study was to evaluate LD50 dose or that dose which would kill more than or minimum half of the animals treated with chemicals to be tested. Based on the initial results of the study, the decision to add more animals for the further study is taken for escalation of dose and to find out the minimum dose that would be lethal to most of the animal taking part in study and also the maximum dose that would be safe for almost all animals in study. Survival is evaluated at scheduled time point i.e. after a week or two weeks, sometimes in time period of a day, after the administration of first dose in multiple dose study,

For determination of MTD of p11 lipoplexes, fixed dose procedure of OECD Organization for Economic Cooperation and Development was used. Typical protocol as stated above includes administration of a drug/drug product in escalating doses through intravenous route and observing animals for any signs of toxicity. Here, the following parameters were evaluated: mortality/morbidity, clinical observations, body weights, food consumption, haematology, organ weights.

Selection of animal species

Healthy young, male and female, either sex, Sprague-Dawley rats (with 8-10 weeks of age), weighing 200-240 g, were used for the study in equal numbers.

Housing and feeding conditions

The temperature in the animal room was kept at 20-25°C. Artificial lighting with the sequence of 12 hr light and 12 hr dark was kept in animal housing. The animals were housed individually. For feeding, conventional rodent laboratory diets were used with an unlimited supply of drinking water.

Preparation of animals

Rats were randomly assigned, marked to permit individual identification, to treatment groups by manual assignment, 2 days before the initiation of dose administration. Animals were excluded from the selection process based on inappropriate body weight.

Preparation of doses

Test substances (lipoplexes) were administered in a constant dose volume of 10 mL/kg by varying the concentration of the dosing preparation. All doses were prepared prior to

administration. All the test substances were sterilized by filtering through 0.2 µ membrane filter prior to administration.

Sighting study

The purpose of the sighting study was to allow selection of the appropriate starting dose for the main study (32). The test substance was administered to single animals in a sequential manner starting from DOSEfirst to DOSElast. The sighting study was completed when a decision on the starting dose for the main study was made (or if a death is seen at the lowest fixed dose).

No of animals and dose levels

- A total of five animals of female sex were used for each dose level investigated.
- The action to be taken following testing at the starting dose level is indicated based on the observations. One of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed dose or test at a lower fixed dose.
- The time interval between dosing at each level was determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose was delayed until there was confidence of survival of the previously dosed animals. A period of 3 or 4days between dosing at each dose level is recommended, if needed, to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response.

Administration of dose

Prior to dosing, all the animals were fasted by withholding food but not water for 3-4 hr. The fasted body weight of each animal was determined and the dose was calculated according to the body weight. The test substances were administered via tail vein of animals using sterile single use disposable polystyrene syringes (BD syringes). In the circumstance that a single dose was not possible, the dose was given in smaller fractions over a period not exceeding 24 hr at 1 hr time gap between two doses and animals were observed for followings listed in table 6.6.

Table 6. 6 Observation of sign for toxicity after intravenous injection

Test	Observation
Mortality/Morbidity	At least once daily
Clinical Observation	2-4 hr after dose administration and daily thereafter.
Body Weights	On Day (prior to dosing), and during the observation period (day 1, 5 and 15)
Food Consumption	Measured per cage for ~24 hr period twice weekly throughout the study
Haematology Parameters	Red blood cell count (RBC) Packed cell volume (PCV) Haemoglobin (HGB) White blood cell count (WBC) WBC differential counts [absolute neutrophil count (ANS), lymphocyte (ALY), monocyte (AMO), eosinophil (AEO), and basophil (ABA) Platelet count (PLC)

Cryo-ultramicrotomy

Very thin section of brain was cut out in order to carry out transportation of formulation across nasal mucosa and brain distribution efficiency of targeted and non-targeted formulations for systemic route using ultramicrotomy. Slow freezing may result in alteration in tissue due to formation of ice crystal formation that exchanges the structure with an effect of "Swiss Cheese" (33). In this procedure, the sample to be freezed is frozen so quickly that water does not get any time to be frozen and stays in a glassy state that does not increase in size when frozen that ultimately results into very thin cut sections of tissues.

In this procedure, ultrachrome fitted in cryochamber can cut very thin sections of 50-60 nm thick (34). Cryo techniques are assemble procedures to obtain stabilised tissues for the observation under microscope. Biological samples are frozen to preserve their morphological structure and Conformation as it exists under normal physiological conditions. These ultrathin sectioning methods using frozen techniques are substitution for the sectioning techniques involving usual Resin fixed tissue sections. The idea is to escape changes in morphology and denaturation effects caused by dehydration. For the observation

under microscope, plasmid was replaced with eGFP as fluorescent emitting plasmid. eGFP's main advantage over conventional fluorescent dyes of the time was the fact that it was non-toxic and could be expressed in living cells, enabling the study of dynamic, physiological processes.

Protocol:

Cryosectioning

1. Tissue was dissected and fixed in 4 % paraformaldehyde for not more than 12 h;
2. After fixation, tissue sample was washed in PBS for 3 times;
3. Transfer this washed tissue sample to 15 % sucrose solution until the tissue sinks at temperature around 4-8 ℃ for 15 minutes;
4. Again transfer it to more concentrated 30 % sucrose solution at same temperature and time mentioned above.
5. Tissue was moved to vials containing 10 % sucrose, 0.04 % Sodium Azide in PBS then stored in refrigerator until cryosectioning takes place.
6. Tissue was placed in cryomold and covered with OCT and allowed to be frozen at -80 ℃ temperature
7. Cut 5-10 micron sized sections and they were placed on slides coated with poly L-Lysine.

Mounting:

1. 80% glycerol + 20% PBS and a pinch of Ascorbic Acid in volume of 50 µL was added to the slide to cover complete section. Caution was taken to dissolve ascorbic acid in PBS before adding glycerol.
2. Cover slip was sealed with nail paint to avoid any drying effects and aluminium foil was used to cover it to elude any exposure to direct light. The sections were observed under confocal microscope after DAPI staining for 10 minutes.

Preparation of solutions

Preparation of pre-fix solution (for transcardial perfusion only)

To prepare 100ml pre-fix solution, add 0.9g NaCl, 0.1g $NaNO_3$, and 1 IU heparin into 80ml water, stir to dissolve. Top up to 100ml with water.

Preparation of fix solution

0.5M phosphate stock solution

To make 1 liter 0.5M phosphate stock solution, dissolve 70.97g of sodium phosphate dibasic anhydrous (Na_2HPO_4, MW=141.96) in 900ml of distilled water, warm to dissolve well, cool to room temperature, top up to 1000ml.

Dissolve 17.33g of sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$, MW=137.99) in 200ml of distilled water, top up to 250ml.

Add above dibasic Na_2HPO_4 solution to monobasic NaH_2PO_4 solution until pH is 7.4, store at room temperature.

To make 100ml fix solution

Weigh 4g paraformaldehyde into beaker, Add 50ml distilled water, heat to 65°C while stirring Turn heat off and continue to stir. Clear the solution with 1-2 drops of 10N NaOH Cool to room temperature. Add 20ml 0.5M phosphate buffer solution. Filter with #1 filter paper Adjust pH to 7.0 with 1N HCl Top up to 100ml with distilled water.

Preparation of post-fix solution

To make 100ml post-fix solution, add 15 or 30g sucrose and 0.1g sodium azide into 80ml fix solution, stir to dissolve. Top up to 100ml with fix solution.

6.11 Transport across the Nasal Epithelia

Rats were divided in two groups (9 rats/group); eGFP solution and liposome containing eGFP were prepared and administrated into rat nasal cavity with 10 µl into each nostril within 10 min interval in individual group. After administration, at the interval of 2 h, 4 h and 6 h, sampling was carried out from nasal cavity of 3 random rats from respective groups at each time points for the presence of fluorescent plasmid which indicates residence time of individual formulation in nasal cavity measured using flourimetry. After completion of residence time study, remaining rats were sacrificed by cervical dislocation for the confocal imaging of nasal mucosa for the presence of eGFP. Rat mucosa was removed for cryosectioning to carry out confocal microscopy. The tissue was embedded in OCT media and cut into 7 µm thickness which was stained for nucleus visibility by DAPI dye which was further analysed by confocal microscope (35).

6.12 Brain distribution study

This study was performed using fluorescent plasmid DNA (eGFP) to observe cDNA across brain to evaluate the effect of different routes and antibody conjugation on distribution in brain. For brain distribution study animals were divided in 5 groups (3 rats per group). For intravenous administration, IGF-II targeted, non-targeted lipoplex formulations and naked fluorescent DNA solution were used; while for the intranasal studies non-targeted lipoplex formulation and naked fluorescent DNA was used. All rats were anaesthetised using ketamine (100 mg/kg) and xylazine (20mg/kg); then formulations containing eGFP was administered. To determine the distribution in brain tissue after intranasal and intravenous

routes, the brains were removed after 4 h of liposomal administration for cryo sectioning. The tissue was embedded in OCT media and cut into 7 µm thickness which was stained for nucleus visibility by DAPI dye, as described under protocol section.

6.14 Animal model for depression

For the generation and evaluating the hypothesis related to clinical studies, animal models are very useful. They have the greatest value as they are useful in imitate clinical circumstances. Depression is a mental condition that has a below par implicated understanding of neurobiology. Current treatments usually do not work for all the patients. Much research is under development to correlate animal model and its progression to the model of human disease.

p11, or S100A10, protein is a member of S100 family of proteins having two calcium binding regions which reported to play an important role in the beginning of depressive disorders by work together with the 5-hydroxytryptamine receptor 1b (5-HTR1b) or 5-hydroxytryptamine receptor 4 (5-HTR4). It is reported that the levels of p11 mRNA and so the level of p11 protein is drastically reduced in depressive rats, mice as well as depressive human patients which can be increased in some of the brain regions with the treatment of marketed anti-depressants. As an example, p11 gene knockout mice who has no potential to generate p11 protein show natural depressive behaviour and a low response or almost no response to 5-HT1b-stimulating agents as the gene to regulate production of p11 protein is totally absent.

Lately, many studies are accomplished that showed the role of the immune system in progression or induction of depression in animal models. Interferon-α (IFN-α) is one of the immune modulator famously used in antiviral therapy and as a part of anticancer treatment regimen. On the other hand, it usually causes CNS side effects such as depression, fatigue, insomnia, anxiety, and cognitive disturbances in chronic use (36). It was found that when IFN-α was used for the treatment of hepatitis C, 80 % patients experienced varied level of depressive disorders (37). Based on these studies, it was hypothesized that IFN-α treatment ended up in inhibition of p11 protein levels and consequent reduction of 5-HTR1b receptor density in brain area associated with CNS dysfunctioning and ultimately, lead to depression.

6.14.1 Interferon α 2B (INF α 2B/ interferon) induced animal models

Selection of dose

Swiss albino mice weighing between 40-60 g were used to induce depression animal model and further efficacy studies. Animal rooms were maintained at 20-25°C and were provided with artificial lighting in the cycles of 12 hr light and 12 hr dark. Individually housed

animals were supplied with unlimited supply of conventional rodent diet and drinking water. Animals were kept in their cages for periods of at least 5 days before dosing sequence was started in order to acclimatize animals with the laboratory conditions and to minimal the environmental effects on animal model.

For the proper selection of dose, mice were divided into 5 groups, (6 mice per group), one of which was control and remaining groups were treated intraperitonially with different doses of INF α 2B (100, 400, 800 and 1200 IU/ day) for 15 days for induction of depression like behavioural despair detected by TST and FST assays (38).

TST and FST assays

Two swim sessions were accompanied for FST studies. A standard 10-min free revelation session, 1 day earlier at the start of the study, to familiarise the mice to the FST; and an end point 6-min session to assess behavioural despair, 24 h after the last INF dose. The FST apparatus was filled with fresh water at ambient temperature. Mice were adjudged to be motionless when they floated in an upright position and completed only slight movements to retain the head above water**.** Mice were tested independently, and the time of immovability was documented in the course of the last 4 min of the 6-min period of exposure, parting the first 2 min for habituation.

The total duration of immobility induced by tail suspension was measured according to the method described by steru et al. as a facile means of evaluating depressing behvaior and antidepressant potential of formulation. Mice were adjudged to be immobile when they floated in an upright position and made only small movements to keep the head above water. Mice were tested individually, and the time of immobility was recorded during the last 4 min of the 6-min period of exposure, leaving the first 2 min for habituation.

6.15 Western blot studies

In general, western blots are a technique that is used to determine the presence of a particular protein from a biological sample. For example, we introduced a plasmid carrying p11 gene, complexed with cationic liposomal career, now to check if mice are actively transcribing and translating gene into desire protein, western blot is one useful tool.

Immunoblotting (Western blotting) is a highly sensitive method for identification of proteins, including antigens of viruses and other plant pathogens. The method is based on combination of gel-electrophoresis and antigen–antibody interaction. High resolution of immunoblotting is provided by combination of electrophoretic separation of proteins, glycol- lipo- and nucleoproteins and the high specificity of antibodies or immune sera used for detection. Under optimized conditions, immunoblotting can detect less than 1 ng of

target antigen in a sample. Western blot analysis can detect one protein in a mixture of any number of proteins. This allows identification of protein of interest from tissue sample.

It is carried out in four stages: (a) separation of the proteins to be analyzed in SDS-polyacrylamide gel (SDS – sodium dodecyl sulfate); (b) Secondly, the separated proteins are transferred electrophoretically ("blotted") onto nitrocellulose to immobilize them. (c) then the primary antibody against the protein of interest is allowed to bind on the membrane. (d) their presence is demonstrated using a radio-labeled or, most commonly, an enzyme-labeled antispecies antibody (secondary antibody against the primary antibody).

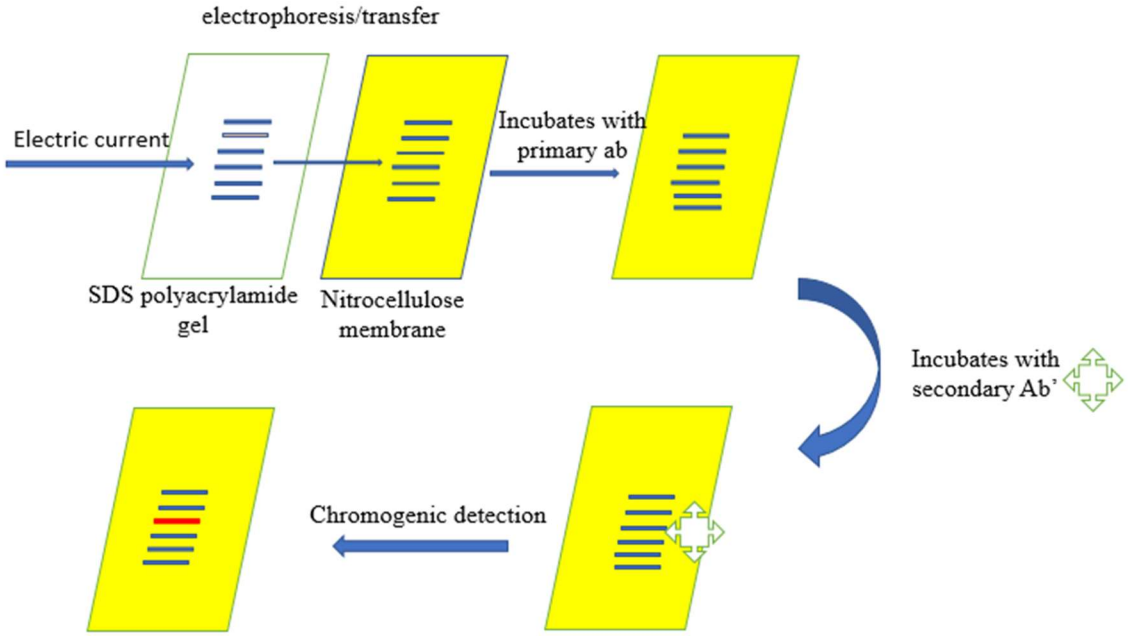

Figure 6. 3 Graphical representation of western blot procedure

Procedure

Young swiss albino mice were divided in 5 groups. Three groups of them received INF α-2B (disease control) and the remaining one received physiological saline pH 7.5 (control). After inducing depression, two groups received treatment of targeted and non-targeted liposomal formulation by i.v. route and one group treated with intranasal liposomes. The leftover group was used as disease control group. Hippocampi of mice treated with saline solution (control), INF a-2B (disease control), liposomal formulation (3 treatment groups)were collected and stored in -80°C until further process. The isolated hippoampi was homogenised in lysis buffer and agitated for 2h. This homogenate was centrifuged at 12000 rmp for 20 min at 4 c and the supernant was collected on ice and total protein conten was determined by Lowery method. Then required volume of sample was mixed with equivalent

volume of 2x Laemmli buffer and heated 95 ℃ for 5 min. 30 µL samples were loaded in SDS-PAGE gel made up of stacking gel (4 %) and resolving gel (12%). 15-20 µl protein ladder was loaded in 1 well to estimate molecular weight. SDS-PAGE was operated at 100 V to allow migration of proteins. After completion, the bands were transferred on nitrocellulose membrane using transfer cassette at 100 V for 2h using ice cold 2 litre transfer buffer and gel packs to maintain temperature. Complete transfer of bands was verified with Ponceau staining. The membrane was blocked using blocking buffer (5-10 ml) for 1 h at 37 c on gel rocker. This blocked membrane was incubated overnight with primary antibody on gel rocker at 4 c. the membrane was washed 4 times with PBST for 15 min. later, it was incubated with antibody for 1 h at 37 c. this was washed 3 times with PBST and PBS for 15 min on gel rocker. The blot was incubated with ECL substrate for 2 min in dark and later observed in chemiluminescence detector. The membrane was washed 4 times with PBST for 15 min and reported with beta-actin antibody which was used as loading control.

6.15 RT-PCR analysis

Effects on p11 mRNA level in brain homogenate was measured using RT-PCR. mRNA expression of p11, was measured for control, disease control and for treatment groups. Treatment group includes targeted lipoplex for systemic delivery and non-targeted delivery for nasal route. P11 mRNA levels were measured on day 1 as control and after 15 days of IFN treatment as disease control. Tissue sample from rat brain was homogenised for that. Total RNA was extracted, quantified and converted to cDNA as per the protocol in chapter 4.

6.16 Result and discussion

6.16.1 Formulation parameters for nasal spray

The US FDA Chemistry, Manufacturing and Controls (CMC) specifications on nasal formulations vouch for assessment of pH value, osmolality and viscosity as an integral part of these product specifications. The pH of nasal cavity will play an important role on the bioavailability of formulation and as per specification, the optimal pH value for the nasal spray formulation is recommended to be between 4.5 and 6.5 (40). Ionization state of liposomes containing cDNA, present in nasal spray would not be affected at spray solution pH, between 6 and 7. Therefore, nasal spray formulated here, was optimised at pH 6.2 using phosphate buffer, as pH too far from the above mentioned range may lead to change in ionization state of liposomes and affect the stability of formulation.

The efficiency of formulation to permeate nasal mucosa can be increased by increasing its residence time on nasal mucosa by optimising formulation viscosity. Viscosity has direct impact on the droplet size distribution, consequently resulted in altered deposition pattern in nasal cavity (41). In this regard, it is important to optimise proper viscosity for the nasal spray formulation. The viscosity of optimised nasal spray with lipoplex dispersed within, was found to be 47 cP which was in accordance with criteria provided by FDA.

Some ingredients present in nasal spray formulation affect the tonicity of formulation, predominantly in the aqueous system. So to balance out this compromising effects, osmotic agents were added to the nasal spray as they adjust the osmotic pressure of formulation such that residence time of lipoplex formulation would increase on nasal mucosa resulting in its more permeation and ultimately increases its bioavailability. Osmolality was determined using osmometer which was found to be around 290mOsm which is hypertonic to the nasal secretion which leads to increase its permeation through nasal mucosa and also in harmony with FDA.

6.16.2 Actuation parameters for nasal spray

Control over the size of the sprayed droplets size is crucial to ensure that deposition in the nose rather than the pulmonary region because droplet size affects the deposition profile and retention time of sprayed particles. Particles larger than 3-10 μm are filtered out by the nose, minimizing the probability of lung deposition (3). Larger droplets may however drip out of the nose after administration whilst too small droplets may deposit in the lower respiratory regions.

For the measurement set-up, the FDA requests to select one or two distances for *in vitro* equivalence purposes in the range of 2 to 7 cm. It is known that the distance between the nozzle and the laser beam affects the DSD measurement, due to different settling velocities of the droplets, the plume dynamics and the varied representation of the true DSD in the measurement zone. External characteristics such as the spray cone angle define the range of spray that exits from the device, while the internal characteristics such as the droplet size distribution help to determine the probability of inertial impaction within the nasal cavity. The influence of actuation distance from 3 to 7 cm was investigated on the DSD and with each increase in actuation distance, growth in D_{10}, D_{50} and D_{90} was observed with a reduction in span value. The plume might get more time to develop at higher distances that would be the reason for it, therefore, droplets, smaller in size can coalesce and results in larger droplets and an overall increase in the droplet size while multiple scattering may be resulted from short distance, owing to the high density of droplets in the measuring zone, which lead to an underestimation of droplet size, hence to circumvent such situations an optimum distance has to be chosen i.e. 5 cm as per results shown in figure 6.4.

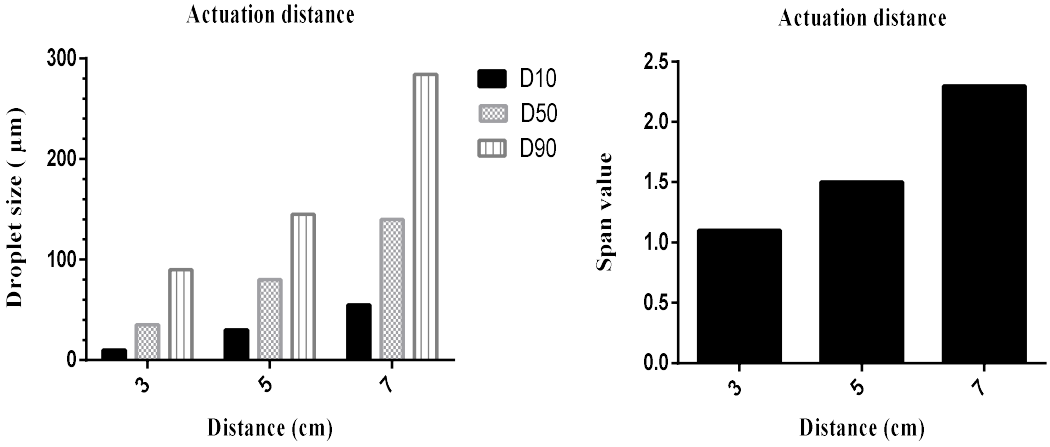

Figure 6. 4 Effects of Actuation distance on Droplet size distribution and Span value

Spraying angle is, one more set-up parameter for DSD measurements besides the actuation distance. Determinations should be performed at a variety of angles even though the most routine measurements are performed at a fixed angle in order to determine position independency of DSD. In this study, the angle of the nasal spray was varied between 0° (horizontal set-up) and 90° (upright position) to conclude a satisfactory angle for repetitive measurements and to assess the position dependency of the DSD. For the 0° and 10° angles, as depicted in figure 6.5, data could not be obtained as spray was not released from the device after actuation. However, no significant change in DSD and span with varying

spraying angles was measured between 20° and 90°. Consequently, the DSD generated by this particular device is not dependent on the position of the nasal spray once a dose is metered. For further studies, an angle of 70°–80° was chosen as this range comes close to in-use conditions when patients administer a nasal spray.

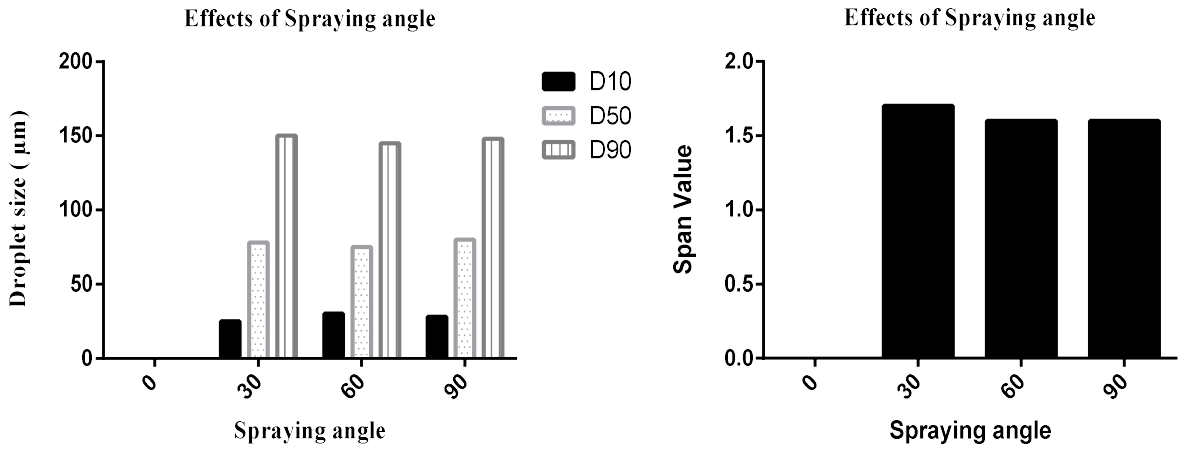

Figure 6. 5 Effects of Spraying angle on droplet size distribution and span value

The effect of stroke length on the DSD was studied and it was determined that stroke length has solitarily a slight effect on DSD in the usual actuation range, but at lower settings, as shown in figure 6.6 with increasing stroke length the D_{50} decreases. In the range of 1–3 mm stroke length, in increasing order, there is a vivid decrease in D_{10}, D_{50} and D_{90} and span, but for the range of 4–7 mm, the DSD and span touch a plateau and persist there. The device is not actuated properly at lower settings, which can be stated by the point that the released mass does not extent the target value and consequently, there is not sufficient energy provided to transform the liquid into fine droplets. However, as soon as the device is actuated with an optimum stroke length i.e. 4 mm, the droplets size rests at constant value.

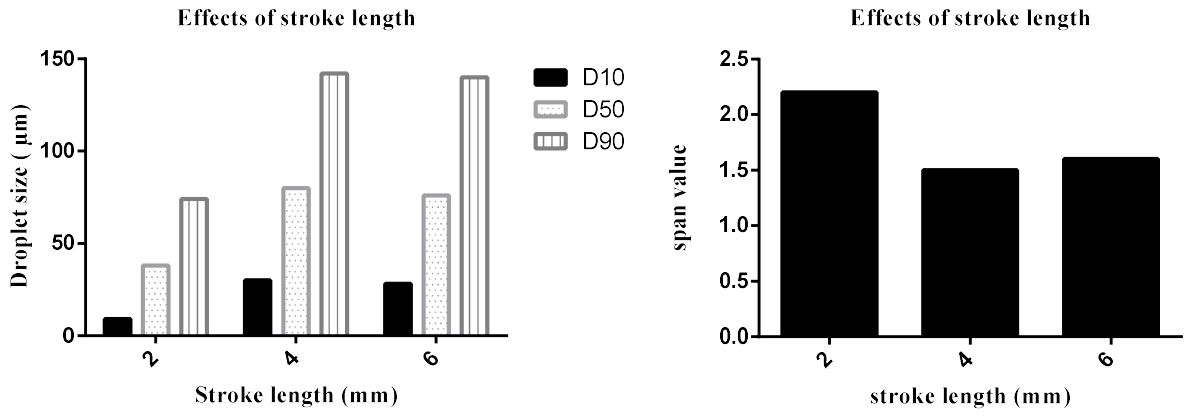

Figure 6. 6 Effects of stroke length on droplet size distribution and span value

The impact of actuation force, in range of 20 to 100 N, on DSD was explored and outcomes for DSD and span are presented in figure 6.7. 20 N was not plenty to actuate the device, and consequently, data could not be gathered for this change. Even for healthy adults, an actuation force of 100 N is too high and not reasonable for DSD measurements, in comparison to actuation forces amid 40 and 60 N. Consequently, data only ranged from 40 to 60 N, was taken into account for further consideration.

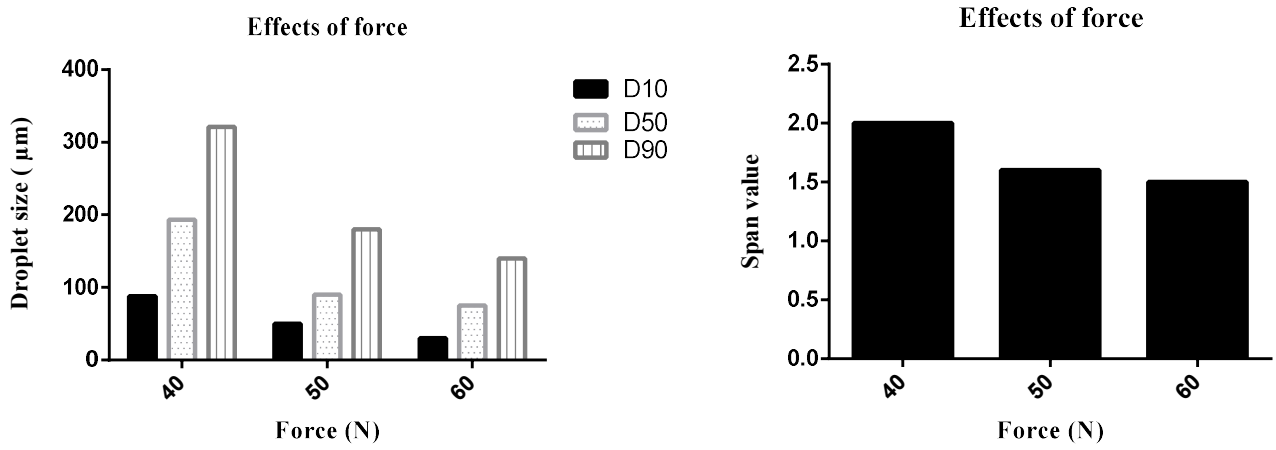

Figure 6. 7 Effects of actuation force on droplet size distribution and span value

After optimizing various instrument parameters like actuation distance (5 cm), spraying angle (70-80), stroke length (4 mm) and actuation force (50 N), DSD measurement was performed, data were analysed and shown in figure 6.8. From data analysis value of D_{10}, D_{50}, D_{90} was found to be 28.42 µm, 77.16 µm, 151.85 µm respectively which is perfectly in ranged with FDA guidelines.

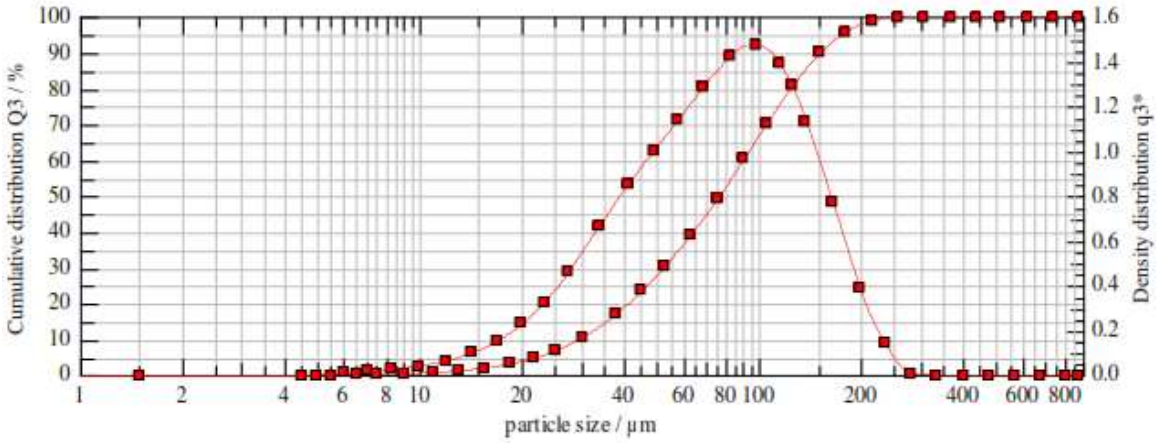

Figure 6. 8 Droplet size distribution of optimised formulation

Newman et al., using gamma scintigraphy, reported that the deposition of a spray with a 35° plume angle gave improved posterior (turbinate) deposition compared to a spray with a similar

droplet size (70 µm) but with a 60° Cheng et al., using the same MRI-derived nasal replica as used in the current studies, also showed that sprays with narrower plume angles and smaller droplet sizes were deposited more posteriorly than those with wider plume angles. Analysis of optimised nasal spray formulation with optimised actuation parameters showed the plum angle of final formulation was found to be 25.5º and 2.54 cm of plume width as shown in figure 6.9 which results in better distribution of droplets in nose resulting in less clearance from nasal cavity and efficient delivery to the brain.

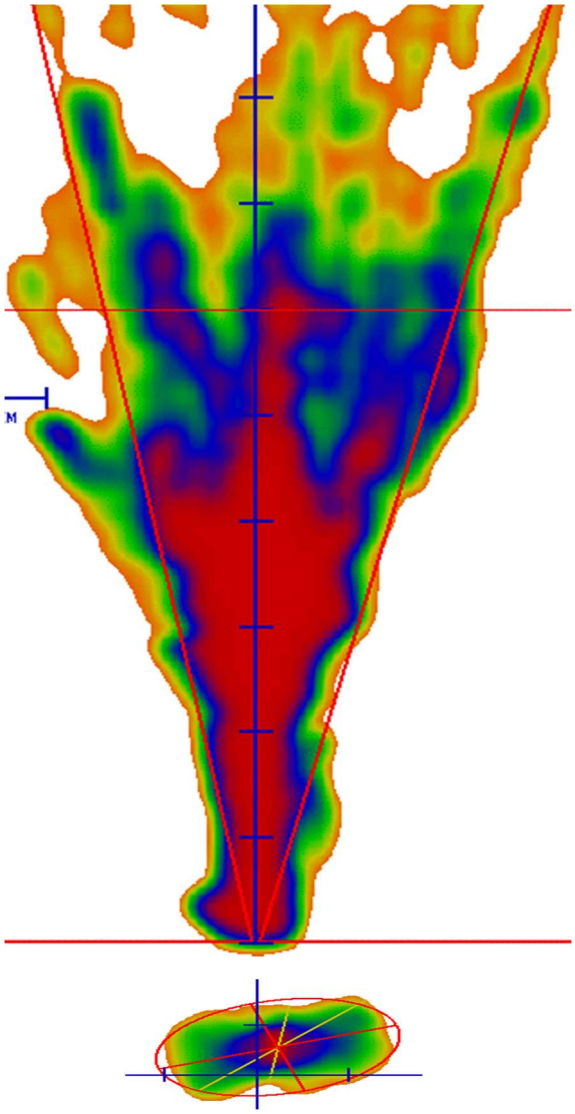

Figure 6. 9 Spray angle and spray pattern of optimised formulation

Table 6. 7 Results of nasal spray characterization

Parameters	Instrument used	Results
pH	Labindia pH meter	6.1-6.2
Osmolality	Osmometer	310 mOsm
Viscosity	Brookfield viscometer	10 rmp: 45-50 cP
Shot weight	-	78.6 mg/actuation
Plume geometry	Spray view	Plume angle: 25.5 º
		Plume width: 2.54 cm
Spray pattern	Spray View	Dmax= 2.55
		Dmin= 1.89
		Ovality= 1.32
Droplet size distribution	Sympatech HELOS	D10= 30.42 µm
		D50= 78.32 µm
		D90= 145.83 µm
		Span= 1.6

Shot weight was found to be 78.6 mg/actuation with % RSD 0.1 which dictates the reproducibility of pump in its actuation capacity which is very important in certain disease conditions where dose reproducibility is an imperative measure.

6.16.3 Ex- vivo nasal permeation study

The effect of DOPE-lipoplex formulation on penetration of nasal mucosa was investigated in comparison to naked cDNA. The penetration of naked cDNA was found to be significant lower as compared to lipoplex formulation after 8 h. The diffusion of macromolecules in the solvent of the intercellular spaces will be reduced with increasing molecular volume (molecular weight) because the diffusion coefficient is inversely proportional to the molecular volume. cDNA being a macromolecule, its penetration across nasal mucosa would be insufficient to cause any pharmacological response. The permeation of cDNA complexed with liposomes formulated from DOPE-A was found to be even better than the control (lipofectamine). This improved penetration of arginine based liposomal formulation was improved might be because of ability of arginine to induce transient widening in cell-cell junctions, resulting in improved transportation of liposomes along with cDNA (macromolecule) through mucosal membrane (42) as depicted in figure 6.10 and table 6.8.

Table 6. 8 Ex-vivo nasal permeation study sheep mucosa

Time (h)	Lipofectamine	DOPE-A	Naked cDNA
	cDNA permeation (%)		
0.5	9.52	9.86	2.24
1.0	21.35	23.04	5.92
2.0	27.81	30.24	8.60
4.0	51.64	52.47	13.71
6.0	72.93	76.21	15.49
8.0	86.29	89.37	19.82

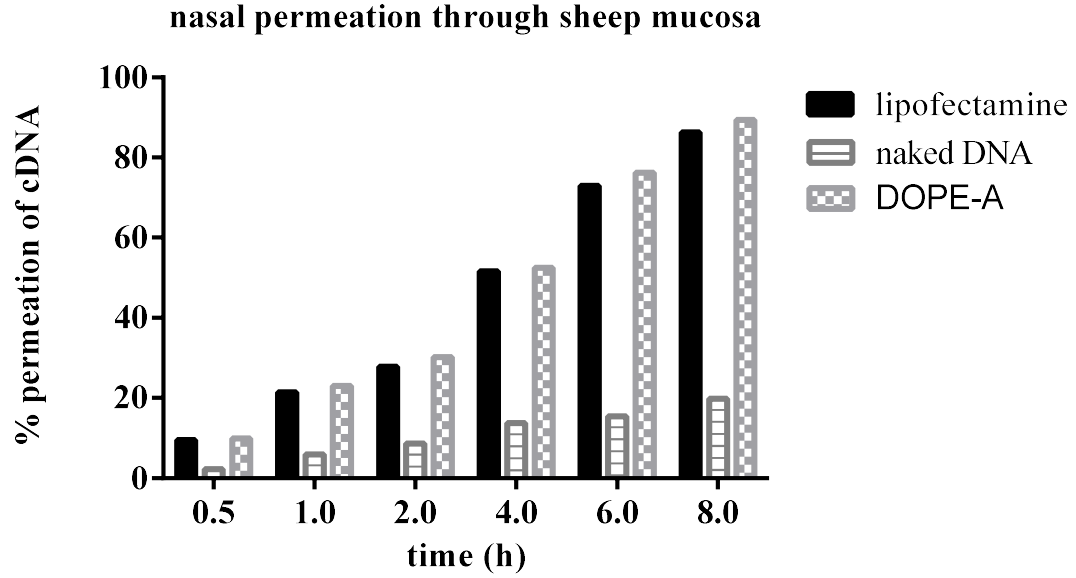

Figure 6. 10 Permeation of cDNA through sheep mucosa

6.16.4 Assessment of local toxicity on nasal mucosa

The safety of intranasal formulations is of crucial importance for medications for chronic therapy. The tissue damage of nasal cavity was evaluated by paraffin section stained with hematoxylin and eosin. As shown in figure 6.11, nasal mucosa treated with PBS pH 7.4 (positive control), the structure of the mucosa was well preserved. The surface pseudo epithelium displayed normal characteristics. After treatment with liposome formulated from DOTAP (negative control), marked alterations in the surface pseudo epithelium were visible; the epithelial lining was completely distracted and they showed lose cohesive, sometimes detached and often vacuolated structure. But after treatment with the liposomal formulation composed of DOPE-A, in nasal spray vehicle, no marked alteration was

observed as compared to negative control from the histological structure. There was also no any evidence of haemorrhage, necrosis and ulceration in treated and untreated nasal mucosa.

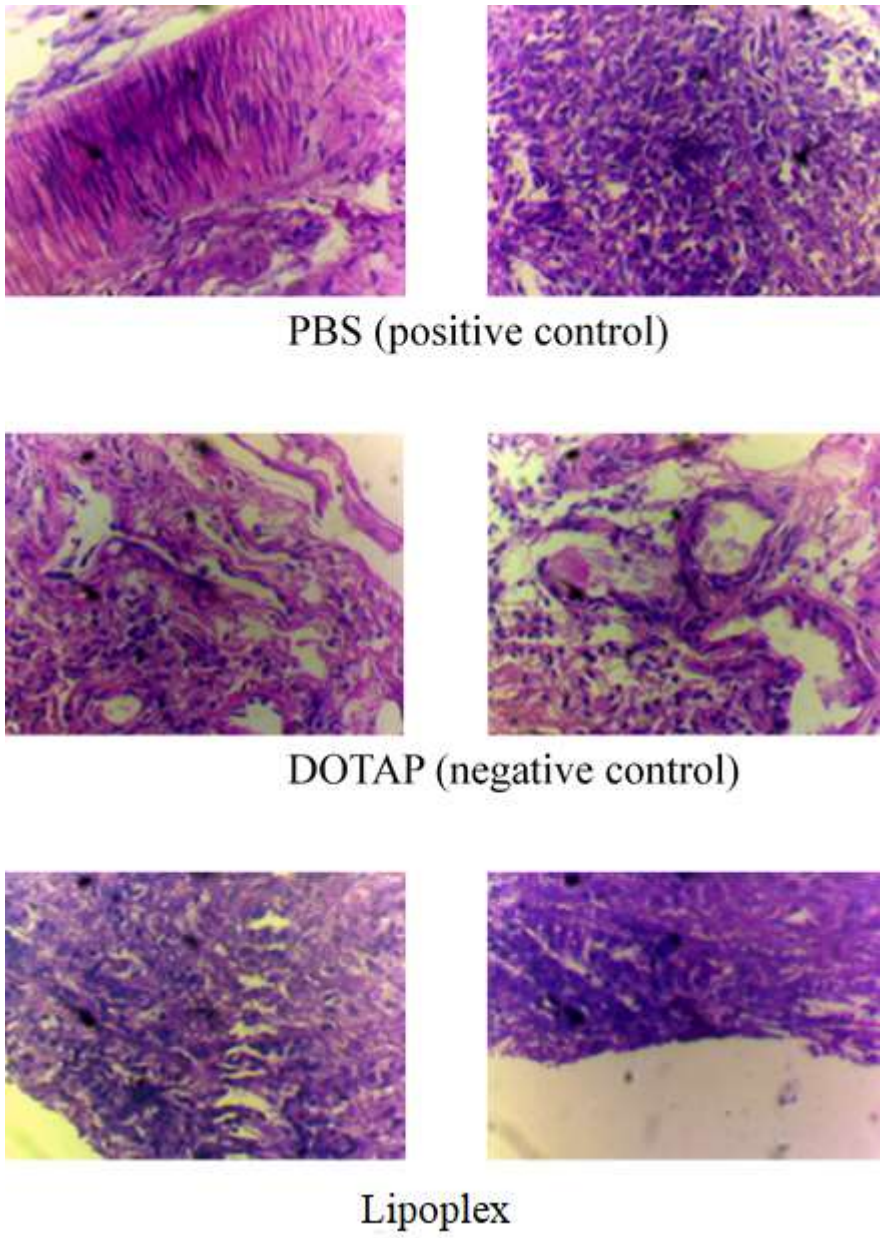

Figure 6. 11 Histopathology of nasal mucosa after treatment with lipoplex

6.16.5 Functionalisation of liposomes

Although pre-insertion method seems relatively easy, there are some drawbacks attributed to this method. In the pre-insertion process, the maleimide functionalised lipid was added to the lipid mixture before the liposomes are formed by hydration with an aqueous phase. The maleimide functionalised lipid gets incorporated into the liposome structure, with a lipid portion occupying the inner bilayer and the hydrophilic part inhabiting the outer aqueous surfaces (43). It also necessitates an additional quantity of functionalised lipid that owing to the high viscosity, marks the process of extrusion tough. In this method, both the inner and outer side lipids are modified and functional lipids inserted to the inner core of vesicles would be wasted in terms of their conjugation efficiency with thiolated mAb. The realization of this problems associated with the conventional PEG-phospholipid incorporation led to a technique of inserting PEG-distearoylphosphoethanolamine (PEG-DSPE) into the outer layer of pre-formed liposomes. In this post-insertion method, functionalised lipids are gradually mixed with dilute liposomal dispersion at temperature approaching glass transition temperature of lipids used in liposomal formation. This interaction between hydrophobic parts of liposomal lipids and functional lipid is one spontaneous process. For the prevention of self-assembly of ampiphilic functionalised lipid, it should be used well below its CMC (critical micellar concentration) value. The biggest advantage of this method that functionalised lipids modify attach only at the outer surface of liposomes, by this means making all the molecules available to attach with monoclonal antibody.

6.16.6 Quantification of sulfhydryl group

The confirmation and differentiation of insertion of meleimide by both the methods was confirmed by ellman's assay as per this calculations:

A 250 µL aliquot of the unknown mixed with 2.5 mL of Reaction Buffer and 50µL of Ellman's Reagent Solution gave an absorbance of 0.879 (after subtracting the blank) using a 1cm spectrophotometric cuvette. Calculate the sulfhydryl concentration in µmoles per mL of unknown. The reported molar absorptivity (molar extinction coefficient, which is expressed in units of M^{-1} cm^{-1}) of TNB in this buffer system at 412nm is 14,150.2 Molar absorptivity, E, is defined as follows: E = A/bc where A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

Solving for concentration gives the following formula: c = A/bE

Absorbance reading for blank test tube = 0.097

Absorbance reading for control = 0.886

Corrected absorbance for control samples= (0.986-0.197) = 0.789

So, c= 0.789/14,150*1=5.576 x 10^{-5} M

This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is 2.50 mL of Reaction Buffer + 0.25 mL of Unknown Sample + 0.05 mL of Ellman's Reagent Solution 2.80 mL of solution

If the concentration of the assay solution is 5.576 x 10^{-5} M, then 2.80 mL of that solution contains

(2.80 mL x 1/1000mL) x (5.576 x 10^{-5}) = 1.561 x 10^{-7}

Similarly, sulfhydryl group in the samples 1 & 2, was estimated that determines the amount of maleimide group available for conjugation with the ligand (mAb), the values were taken into account by subtracting the test samples thiol concentrations from the absorbance of blank samples and results are depicted in table 6.9.

Table 6. 9 Absorbance and concentration of sulfhydryl group present in antibody containing samples

Sample	Absorbance (n=3)	Concentration (moles)
Blank	0.097 ± 0.009	-
Control	0.886 ± 0.135	1.561 x 10^{-7}
Sample 1	0.642 ± 0.086	1.078 x 10^{-7}
Sample 2	0.319 ± 0.073	0.439 x 10^{-7}

6.16.7 Characterization of immunoliposomes

SDS-PAGE analysis

In present work, anti-IGF-II monoclonal antibody was conjugated to DOPE-A liposomes CNS targeted delivery for depression. Traut's regaent was used for the thiolation of mAB (mAb-SH) to complete conjugation reaction with maleimide linker attached to liposomes. Reduction of mAB to mAb-SH may take place during experiments when samples are heated with loading buffer containing a reducing agent may give a false interpretation of results and thus, to avoid such situation, non-reducing condition was used for SDS-PAGE analysis. Buffer containing 1 mM EDTA was used throughout the procedure in preparation of samples to avoid metal ions catalysed oxidation of reduced sulfhydryl group to disulphide.

Coomassie staining method was used for detection of protein after SDS-PAGE analysis (44). This protein-determination method involves the binding of Coomassie brilliant blue G-250 to protein. The protonated form of Coomassie blue is a pale orange-red colour whereas the unprotonated form is blue. When proteins bind to Coomassie blue in acid solution their positive charges suppress the protonation and a blue colour results. The assay is very reproducible and rapid with the dye-binding process virtually complete in ~2 min with good colour stability. The results of SDS-PAGE after coomassie staining was shown in figure. As represented in figure 6.12, Lane 1 is a standard protein marker showing bands with a known molecular weight. In lane 2, standard solution of mAb was loaded showing band around 30-35 kDa. No other bands in this lane confirming purity of mAb showing no degradation of antibody. Lane 3 and 4 respectively showing bands for antibody conjugated to liposomes after pre-insertion and post-insertion technique.

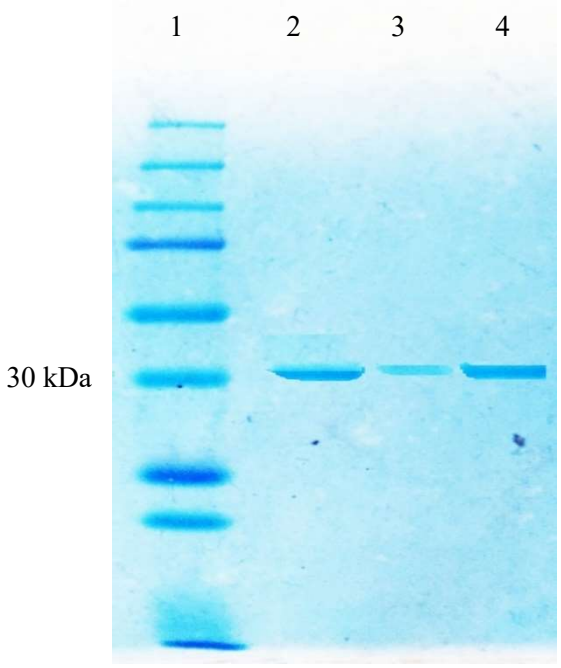

Figure 6. 12 Coomassie staining of IGF-II conjugated liposomal formulations

Bradford's method

Calculation of ellman's assay showed more functionalisation of liposomes for post insertion technique, further evidenced by high density bands of mAb for post insertion technique proving more functionalisation resulting in more conjugation of mAb to liposomes. The mAb concentrations in unknown samples of pre-insertion and post-insertion technique was measured using Bradford's method. In a way, it was an indirect method to estimate conjugated mAb to liposome as unconjugated portion of mAb was removed by sepharose column. The immunoliposomes were prepared by 20 µg quantity of mAb and then incubation was carried out at 1:50 weight ratio of mAb to functionalised lipid. The amount of mAb on pre-insertion and post-insertion functionalised liposome was found to be around 16.9 µg and 11.4 µg. thus, % mAb conjugation over liposomal surface was found to be 84.5 % for post-insertion method and 57.0 % for pre-insertion method.

Surface modifications of lipoplex by mAb changed the complexation efficiency of liposomes to a certain ratio. Steric hindrance by antibody molecules around liposome vesicles might be the reason decreased complexation efficiency or same complexation at higher N/P ratio as shown in figure 6.13.

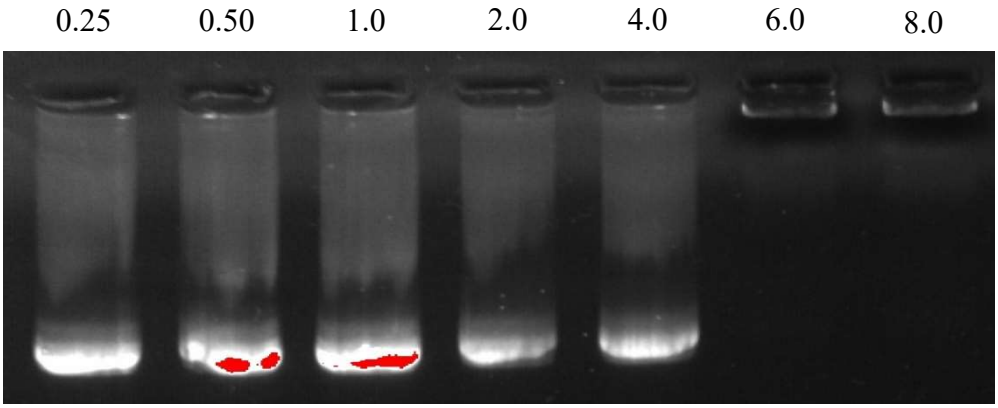

Figure 6. 13 Complexation efficiency of IGF-II targeted DOPE-A lipoplex

Moreover, mAb being a heavy molecule surfaced on liposomes, it affects the mobility of liposomes, dropping the chances of approaching negatively charged cDNA molecule to complex. The physicochemical properties of the lipoplexes were evaluated for the mAb conjugated lipoplexes. It was observed that the particle size of the lipoplexes changed after conjugation as shown in figure 6.14; however, zeta potential of the lipoplexes was not changed drastically for the lipoplexes.

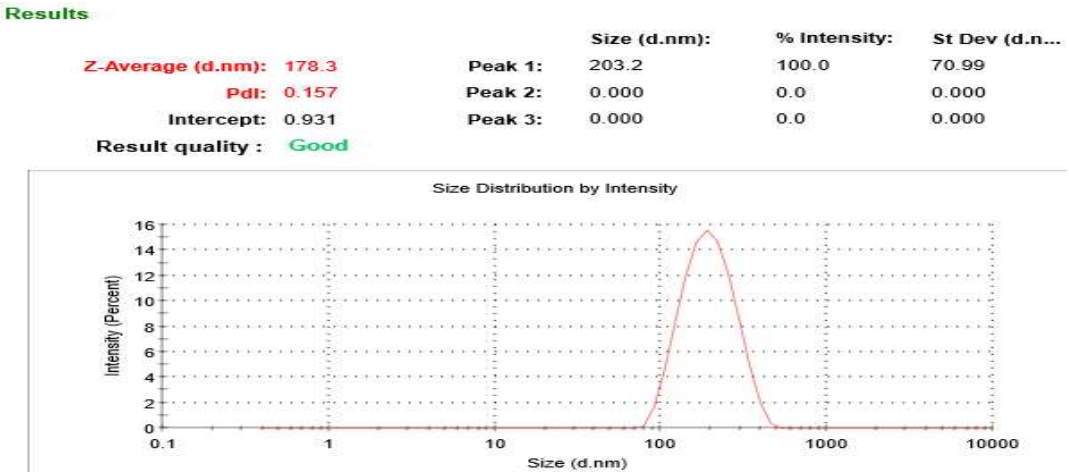

Figure 6. 14 Size of IGF-II targeted lipoplex

6.16.8 Cell cytotoxicity and cell uptake study

Cytotoxicity studies of the targeted lipoplexes were carried out to compare their cytotoxicity against the non-targeted lipoplexes prepared without targeting mAb. It was observed that there was no significant difference in the cytotoxicity of the lipoplexes before and after conjugation to the targeting mAb as represented in figure 6.14.

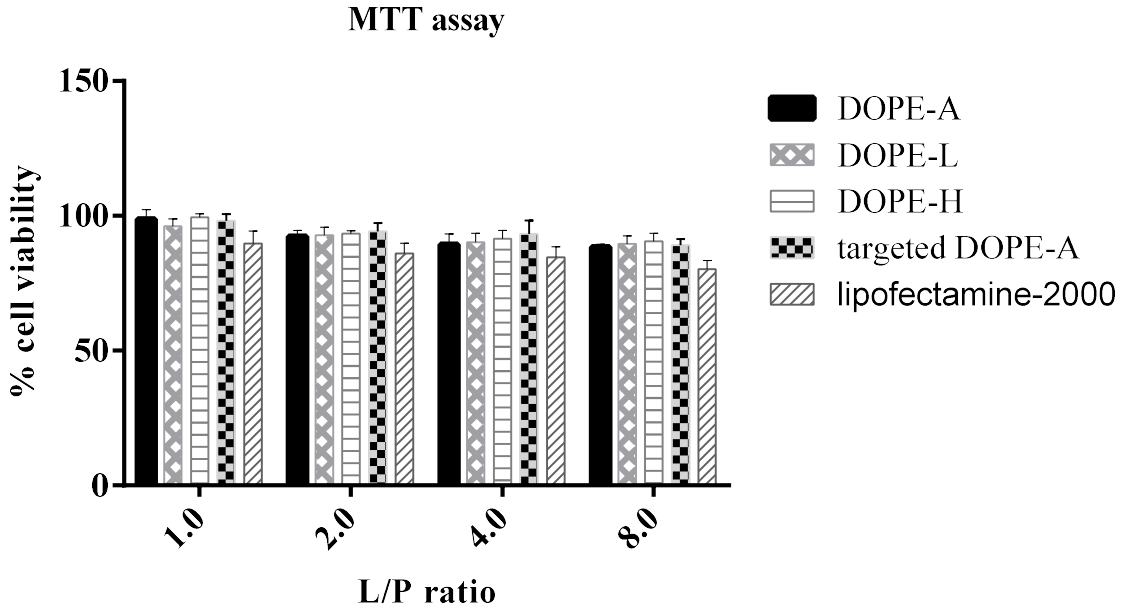

Figure 6. 15 Cytotoxicity of targeted and non-targeted lipoplex formulations

Qualitative cellular uptake of targeted formulation was carried out to see the effects of antibody conjugation on the cellular internalization. From the results, it was observed that

the cellular uptake of targeted formulation changed drastically after mAb conjugation to the same formulation.

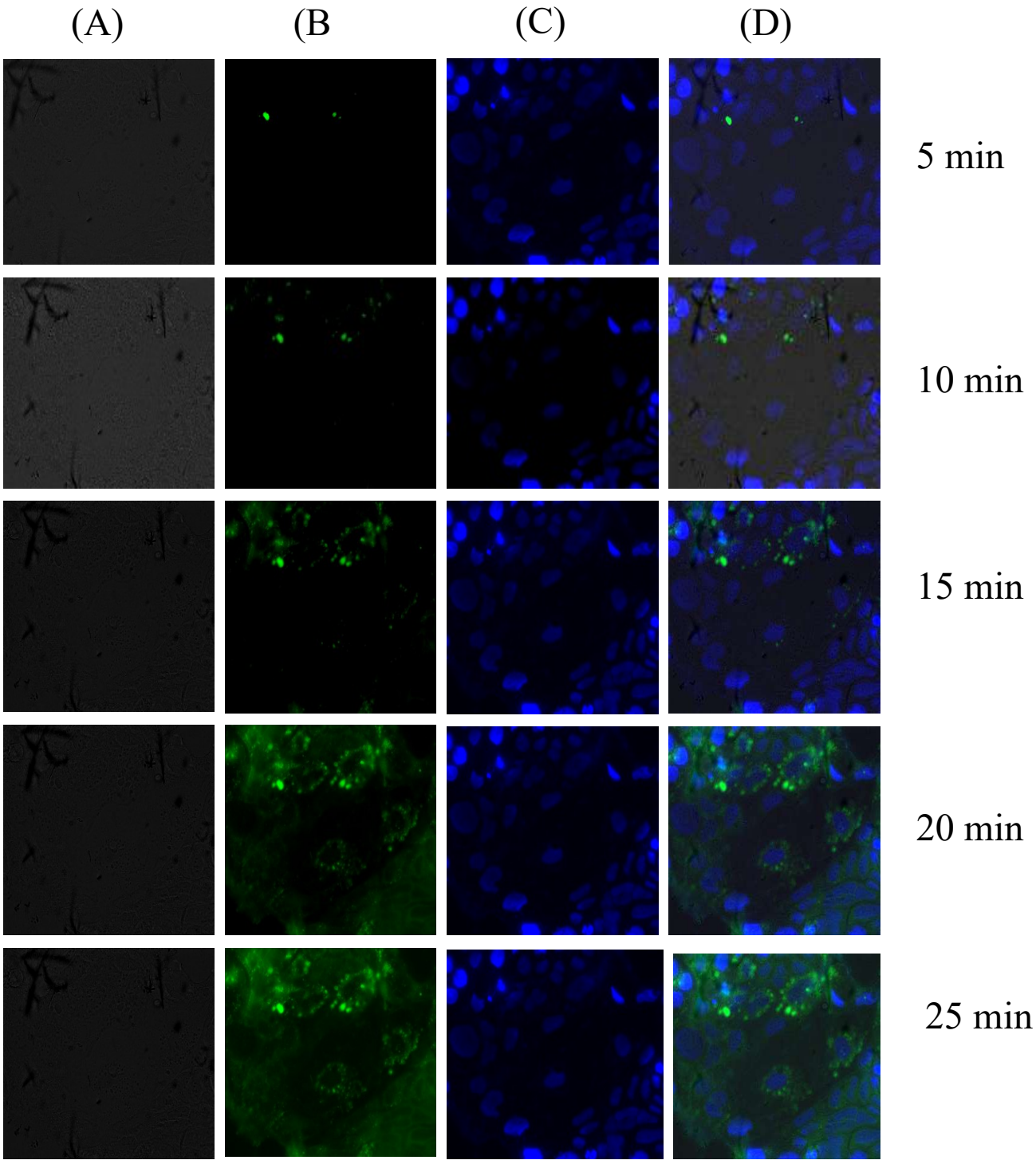

Figure 6. 16 Live imaging of SHSY5Y cells using confocal microscope after treatment with IGF-II targeted DOPE-A lipoplex

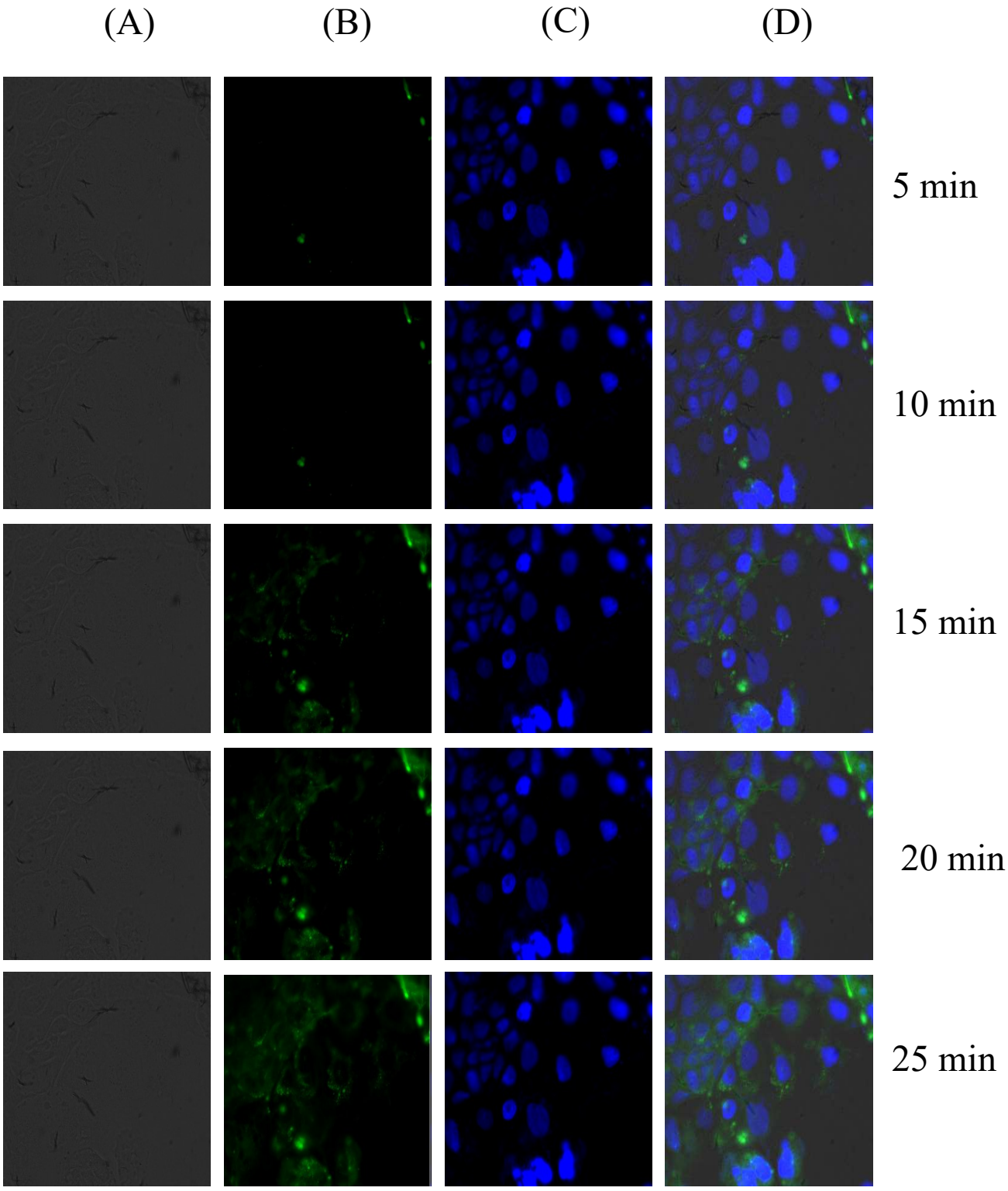

Figure 6. 17 Live imaging of SHSY5Y cells using confocal microscope after treatment with DOPE-A lipoplex

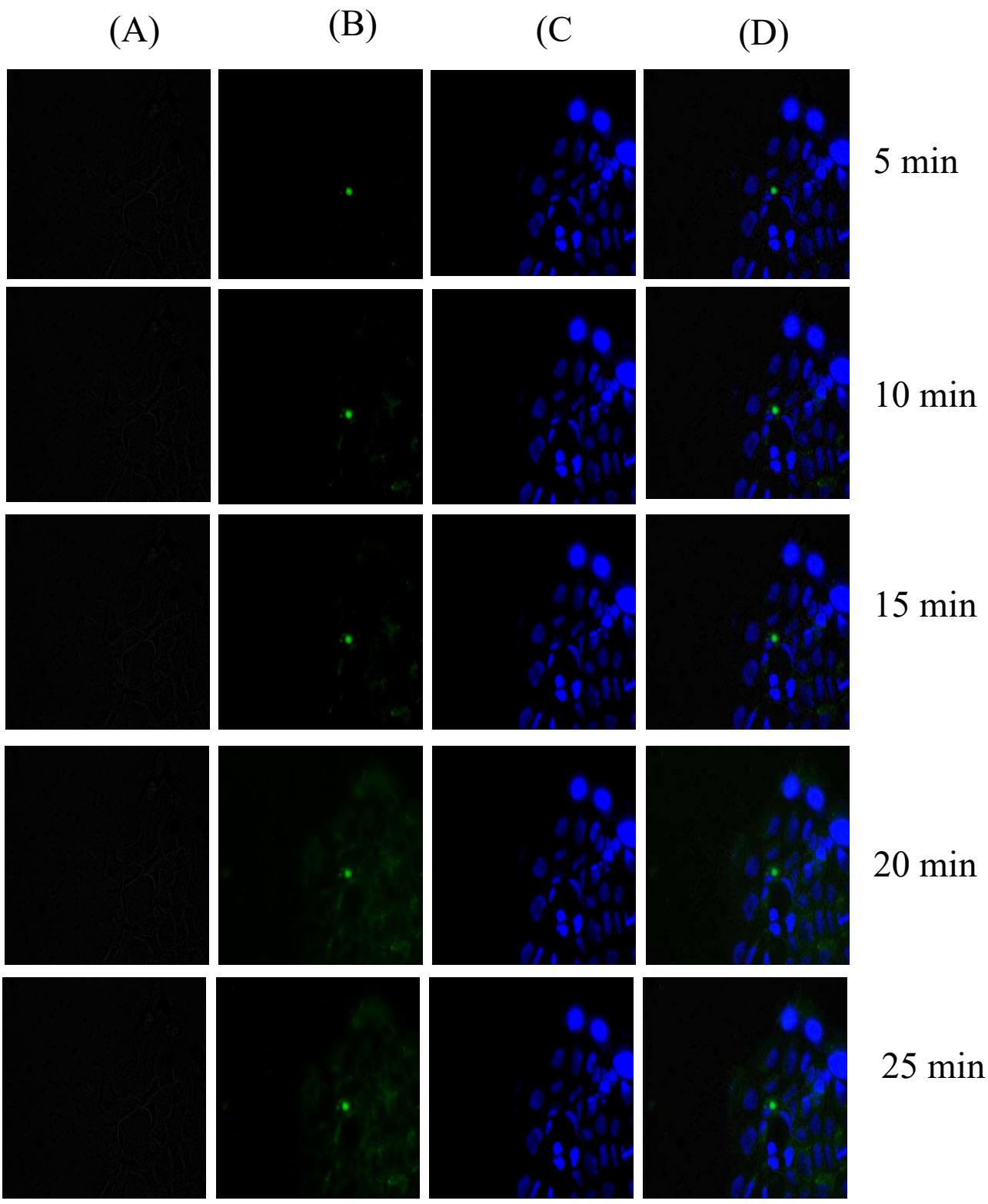

Figure 6. 18 Live imaging of SHSY5Y cells using confocal microscope after treatment naked cDNA

Live imaging was performed for 25 min span after treatment with different targeted, non-targeted and naked cDNA formulations and show in figure 6.15, 6.16 and 6.17. Initial images showed less florescence compared to images of later stages. At any stage, non-targeted formulation showed less fluorescence or less uptake of formulation in comparison to targeted formulation and the same way, naked cDNA formulation showed less fluorescence compared to both targeted and non-targeted formulations. Moreover, images also showed naked cDNA remained outside of the cell surface while liposomal formulation found to enter cell bilayer easily and more efficiently. As discussed before, fusogenic property of DOPE and arginine plays role in macropinocytic transfer of formulation through the bilayer. Targeted formulation was formulated from arginine modified DOPE lipid with IGF-II mAb as targeting moiety. Cellular uptake experiment was conducted in SHSY5Y which contains IGF-II receptors that played an important role in engulfing more formulation, resulting in more fluorescence at any given time in comparison to naked cDNA and non-targeted formulations (45).

6.16.9 Intravenous acute toxicity study

Targeted and non-targeted lipoplex formulation containing p11 cDNA were administered intravenously to the female Swiss Albino mice as per the dosing protocol given in table 6.10 for sighting study. During sighting study, formulations were administered to a single mice for each dose level and observed for signs of toxicity for 24 hr.

Table 6. 10 Sighting study for dose selection in swiss albino mice

Group	**Dose of lipoplex equivalent to**		
Normal saline	-	-	-
Non-targeted lipoplex	100 µg/kg of cDNA	250 µg/kg of cDNA	500 µg/kg of cDNA
Targeted lipoplex	100 µg/kg of cDNA	250 µg/kg of cDNA	500 µg/kg of cDNA

All animals were found healthy and no signs of any toxicity were observed. Results for sighting studies are summarized in table 6.10.

Table 6. 11Mortality study in Swiss albino mice at varied doses

Formulation	Dose	mortality
Normal saline	500 µg/kg of pDNA	none
	250 µg/kg of pDNA	
Non-targeted lipoplex	500 µg/kg of pDNA	
	250 µg/kg of pDNA	
Targeted lipoplex	500 µg/kg of pDNA	
	250 µg/kg of pDNA	

After performing the sighting study, 250 µg/kg of cDNA and 500 µg/kg of cDNA were considered for the main study. Four animals were administered with the selected doses of cDNA making up total of 5 animals per group including one animal from sighting study and results are shown in table 6.11.

Table 6. 12 Average change in weight prior and during study (gram)

Period	Normal saline	non-targeted lipoplex	Targeted lipoplex
Day 1	+ 2.9	+1.2	-0.5
Day 5	+3.1	+1.0	+0.7
Day 15	+2.7	+0.9	+1.2

As shown in table 6.12, there was no significant difference between weights of saline treated animals and lipoplex treated animals.

Table 6. 13 Feed consumption by group per cage (gram)

Period	Normal saline	Non-targeted lipoplex	Targeted lipoplex
Prior to study	15.5	15.6	17.2
Day 1	12.9	14.4	16.9
Day 5	15.1	16.2	17.0
Day 15	14.3	15.8	17.6

As shown in table 6.13, there was no significant difference present in the food habit of saline and lipoplex treated animals.

Table 6. 14 Results of CBC study after intravenous injection of formulation

Tests (with units)	Normal range	Saline	Non-targeted lipoplex	Targeted lipoplex
Haemoglobin (gm/dl)	13.5-18.0	14.8	14.1	14.4
RBC (millions/mm^3)	6.76-9.75	9.00	7.54	8.19
PCV (%)	37-50	43.1	47.2	40.4
Total WBC count (mm^{-3})	6600-12600	7596	8532	12293
Differential count (% of WBC)				
Neutrophils	60-70	65	64	62
Lymphocytes	20-30	25	29	29
Eosinophils	01-04	02	03	04
Monocytes	02-06	03	04	04
Basophils	00-01	00	00	01
Platelets (ml^{-1})	150000-460000	390000	290000	200000

Monoclonal antibodies are important therapeutic tools, but their usefulness is limited in patients who experience acute infusion reactions, most of which are consistent with type I hypersensitivity reactions including anaphylaxis. The use of monoclonal antibodies (mAbs) in modern medicine is a technological advance that has revolutionized our approach for

treating and thinking about a number of human diseases. By targeting specific proteins, mAbs provide efficient option for the treatment of cancer and CNS disorders like in our case IGF-II providing an option for CNS targeting. Most reactions to mAbs occur acutely during the infusion, with symptoms ranging from mild rigors to systemic anaphylaxis. CBC (complete blood count) was performed to check for the toxic reactions to blood components by IGF-II targeted lipoplex formulation and non-targeted lipoplex formulation as mAb being a foreign protein for our body. As per the results shown in table, all the blood components including haemoglobin, WBC, differential WBC, RBC and platelet counts were within their normal range, showing no sign of any allergic reaction as depicted in table 6.14.

6.16.10 Transport across nasal mucosa

Transportation studies performed by *in vivo* imaging allowed us to follow the cDNA distribution across nasal epithelia (46). It was observed that after 1.5 h the naked cDNA in the form of eGFP solution, had totally been disappeared from the nasal area, while liposome formulated cDNA was still present after 8 h in measurable quantity which depicts the longer residence time of cDNA entrapped in liposomal formulation in comparison to naked cDNA formulation suggesting degradation across nasal mucosa or faster adsorption of naked cDNA through nasal cavity. But as per figure 6.18, confocal microscopy clearly showed more fluorescence in case of liposomal formulation than plain solution confirming more transportation of liposomal entrapped cDNA through nasal mucosa while the counter part was possibly washed away by nasal secretion or degraded by enzymes present in nasal cavity.

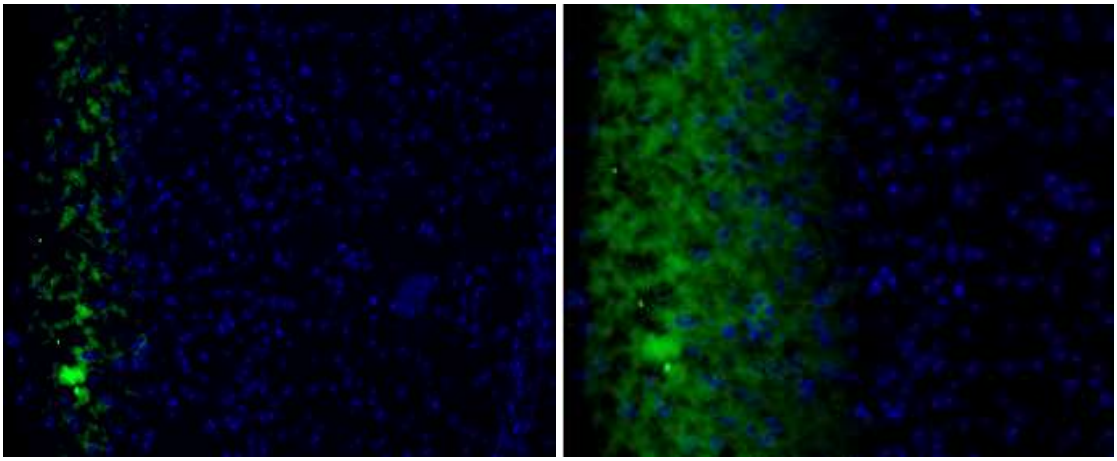

Figure 6. 19 Confocal images of cryo-sectioned nasal mucosa after intranasal treatment with naked DNA and lipoplex formulation

6.16.11 Brain distribution study

A large number of compounds of pharmaceutical interest are tested nowadays for the treatment of neurodegenerative disorders. However, most of these compounds which demonstrate efficacy *in vitro* are not able to reach the central nervous system (CNS), at least not in pharmacologically significant concentrations. Delivery of therapeutic agents to the CNS is hindered by the blood–brain barrier (BBB), which is composed of a tightly sealed layer of endothelial cells and astrocytes, regulating the permeation and diffusion from the blood stream into the brain. Therefore, for any compound affecting CNS disorders, its distribution across brain is very vital to help cure disease. Brain slices of 7 μm thickness were imaged in more detail by confocal microscopy, in order to localize the fluorescent DNA in its solution and lipoplex form at cellular level. The distribution across brain tissue was visualised from the signal of eGFP. DAPI was used to stain the nuclei. The confocal fluorescence images (Figure 6.19) confirmed the difference in intensity of fluorescent DNA across brain tissue, for both systemic and nasal route; as well as difference in distribution patterns of lipoplex formulation and cDNA in its solution form.

As shown in figure 6.19, during the systemic administration of formulations, both the targeted and non-targeted lipoplex formulations were able to cross the blood–brain barrier and accumulate in the various areas of brain. Lipoplex formulations, both targeted and non-targeted, showed more accumulation of cDNA in brain, in comparison to solution form, confirming the more permeation of lipid based formulation though BBB. More intense fluorescence for IGF-II targeted lipoplex formulation confirmed the role of IGF-II receptors present in BBB for transportation of targeted formulation through BBB. For intranasal route, size of the molecule passing through nasal mucosa and nasal clearance plays an important role in its distribution to the brain. Moreover, to accumulate in brain through nasal route, olfactory region helps in bypassing BBB, so there is no role for receptor mediated transcytosis to play for this particular route. Even though lipoplex formulation showed more accumulation of cDNA in brain than that of solution form it showed less intense fluorescence in comparison to its systemic delivery.

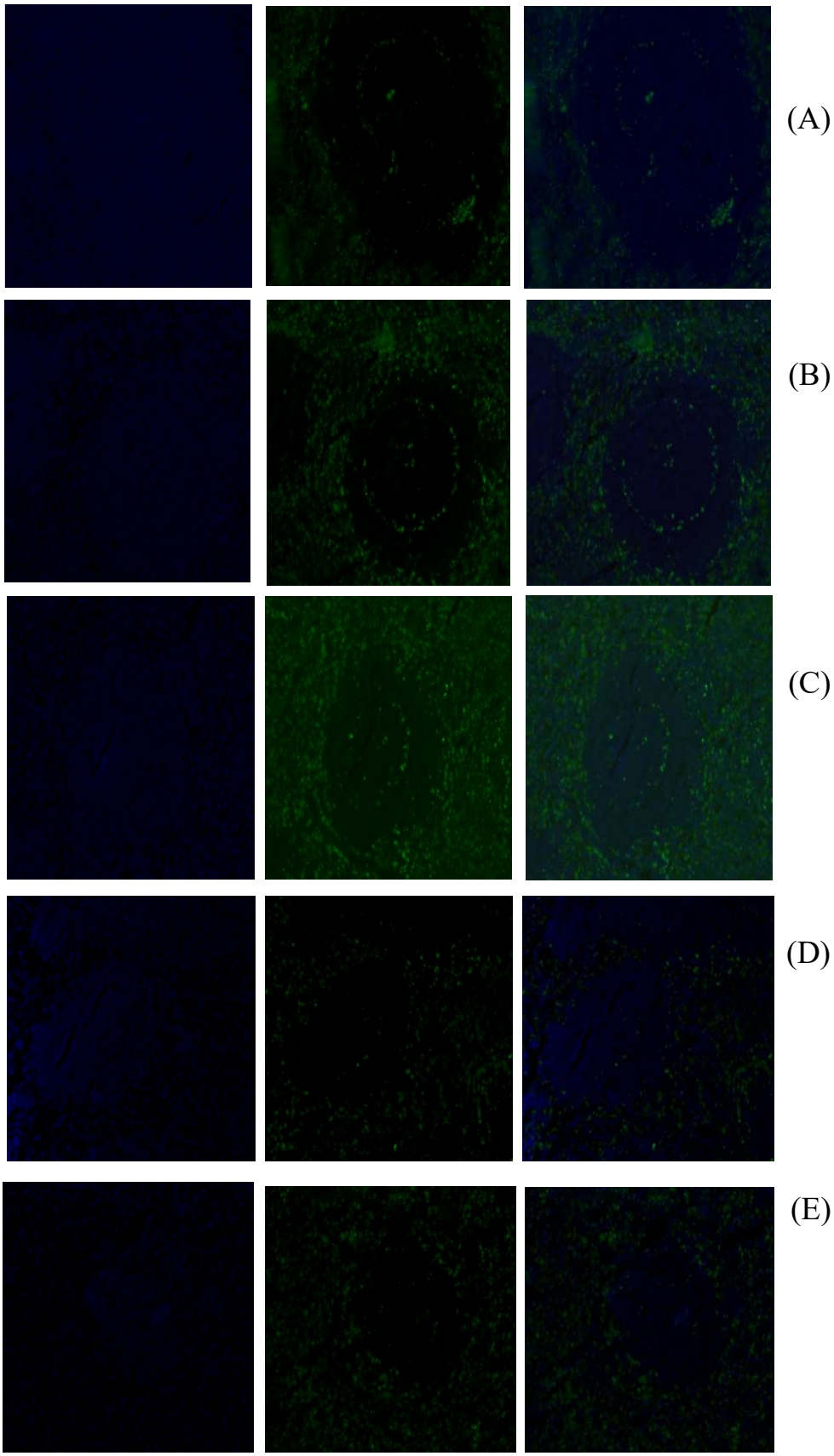

Figure 6. 20 Confocal images of cryo sectioned brain tissue after treatment with (A) i.v. naked DNA, (B) i.v non-targeted lipoplex, (C) i.v. targetd lipoplex, (D)intranasal delivery of naked DNA, (E) intranasal delivery of lipoplex

6.16.12 Animal model for depression

TST and FST assays are commonly used as depression-related behavioral paradigms, as time of immobility in each assay is increased by treatment with sufficient dose of INF. Mice treated with normal saline showed immobility time of around 4 min which was considered to be standard response time for further studies. Mice treated with INF up to 800 IU/day showed no change in immobility time showing no sign of disease progression but from the dose of 1200 IU/day, showed progression of disease concluded by increase in immobility time. Therefore, the dose of 1200 IU/day was selected as minimum dose required for the induction of depression like behaviour.

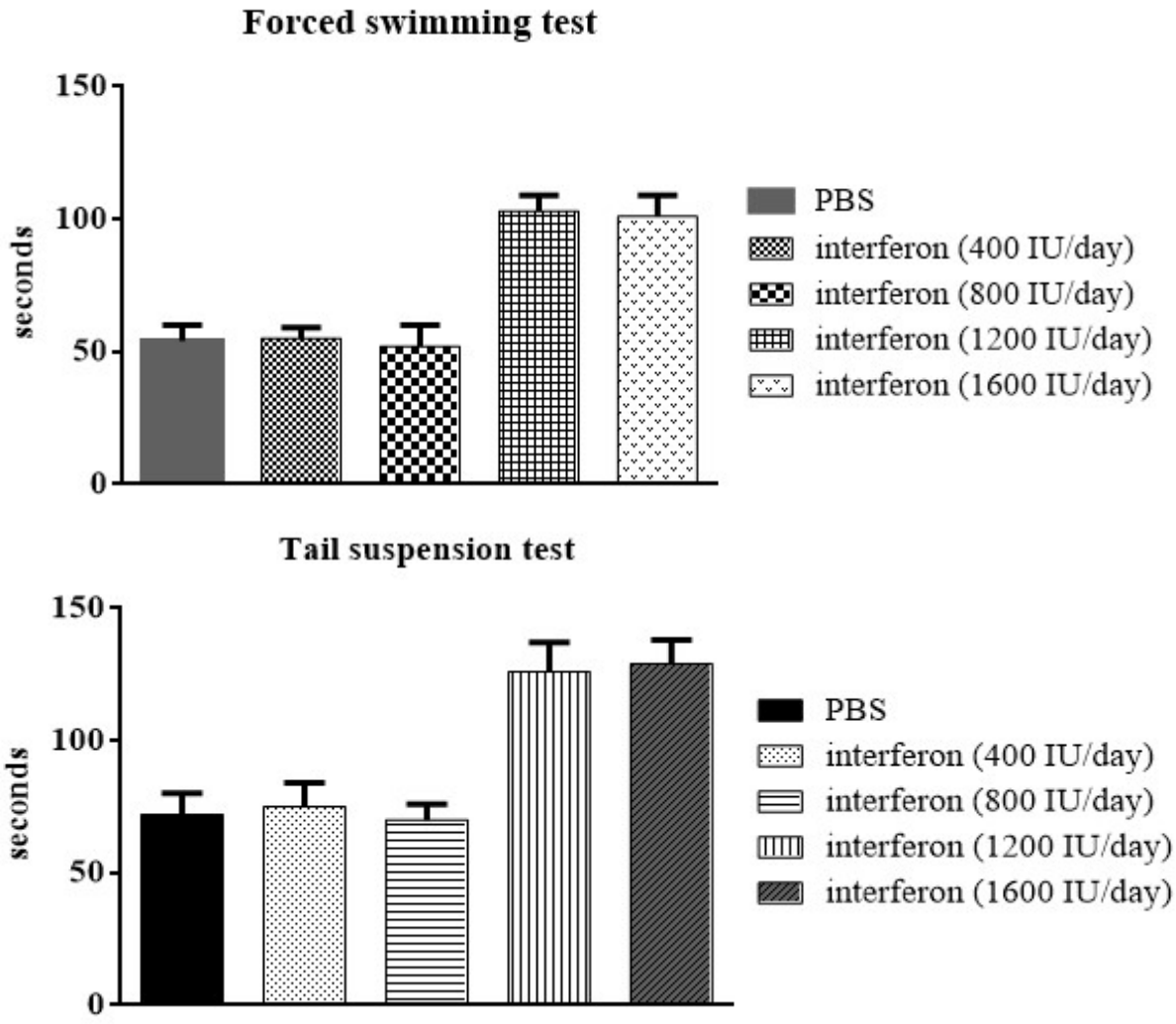

Figure 6. 21 Dose dependency of Interferon induced depression model

the As shown in figure 6.20, after treatment with interferon up to 800 IU/ day there was no noticeable change in immobility time for any of these tests indicating no change in the behaviour that could sign towards depressive disorder. But the from the dose of 1200 IU/day there was drastic change in the immobility time, around 105 seconds for FST and 123

seconds for TST; representing change in the response of animals, indicating towards their depressive behaviour.

6.16.13 Western blot

It is not uncommon for IFN-α treatment to induce the onset of a severe mental disorder, particularly major depression. The mechanisms underlying this process remain unclear, but several hypotheses suggest that IFN-α-induced depression may be related to the effects of IFN-α on tryptophan metabolism, the synthesis of 5-hydroxytryptamine, and/or the activity of serotonin reuptake receptors reducing p11 protein level (47). To investigate the effect of interferon on p11 protein was analysed using western blot studies. Our study suggested that interferon reduced the level of p11 protein around 70.52 % in brain hippocampus which is clearly visible in figure 6.21.

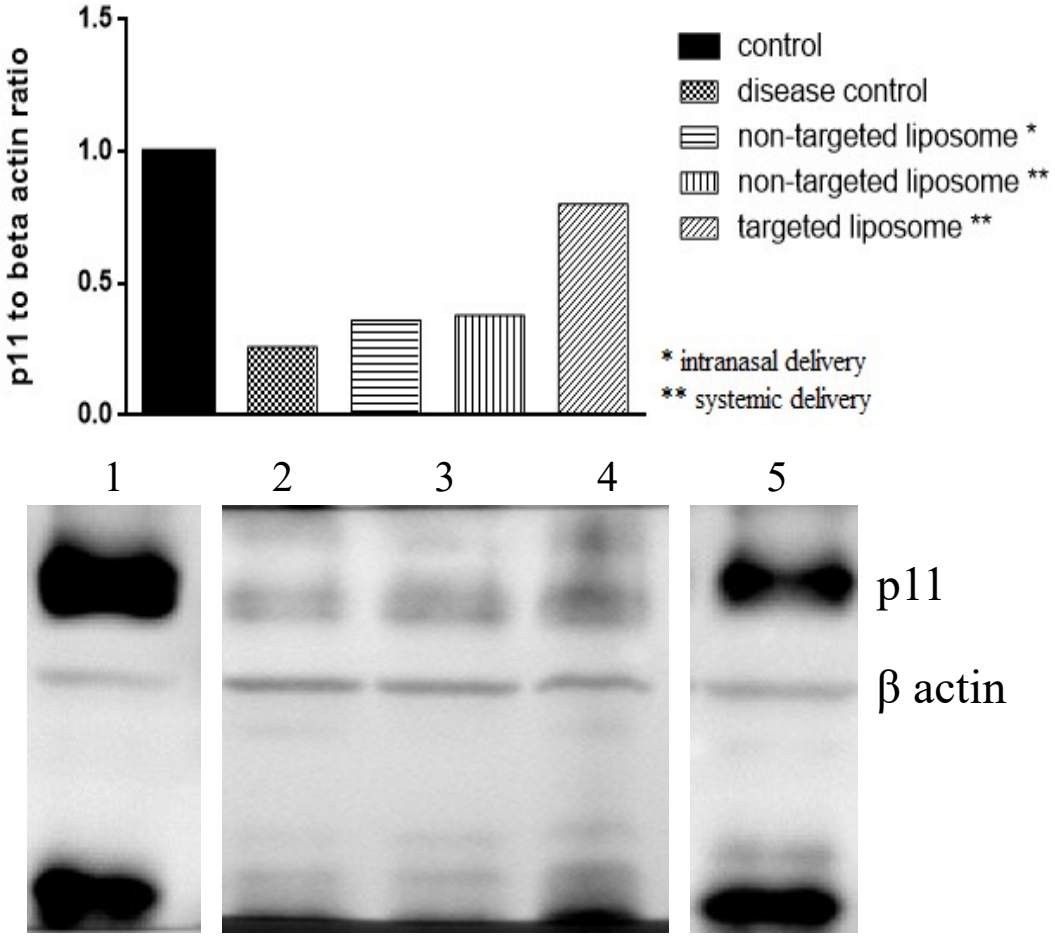

Figure 6. 22 Western blot analysis of p11 protein in animal brain tissue

This study suggested that the IFN-α administration resulted in depression-like behavior in mice and inhibited the protein levels of p11. Furthermore, after the systemic treatment with IGF-II targeted liposomal formulation p11 protein level was found to be around 79.85 % in comparison to non-targeted systemic delivery showed p11 protein level around 42.91 % while same formulation through intranasal delivery showed 41.26 % of p11 protein level. IGF-II targeted liposomal formulation improves the protein level mostly because of its superior ability to accumulate in brain i.e. site of action in our case, proved by previous studies. In a way, results from western blot studies supported the results obtained from brain distribution studies.

6.16.14 RT-PCR studies

The reduction in p11 mRNA levels after 15 days treatment of Interferon is a sign of interferon induced depression.

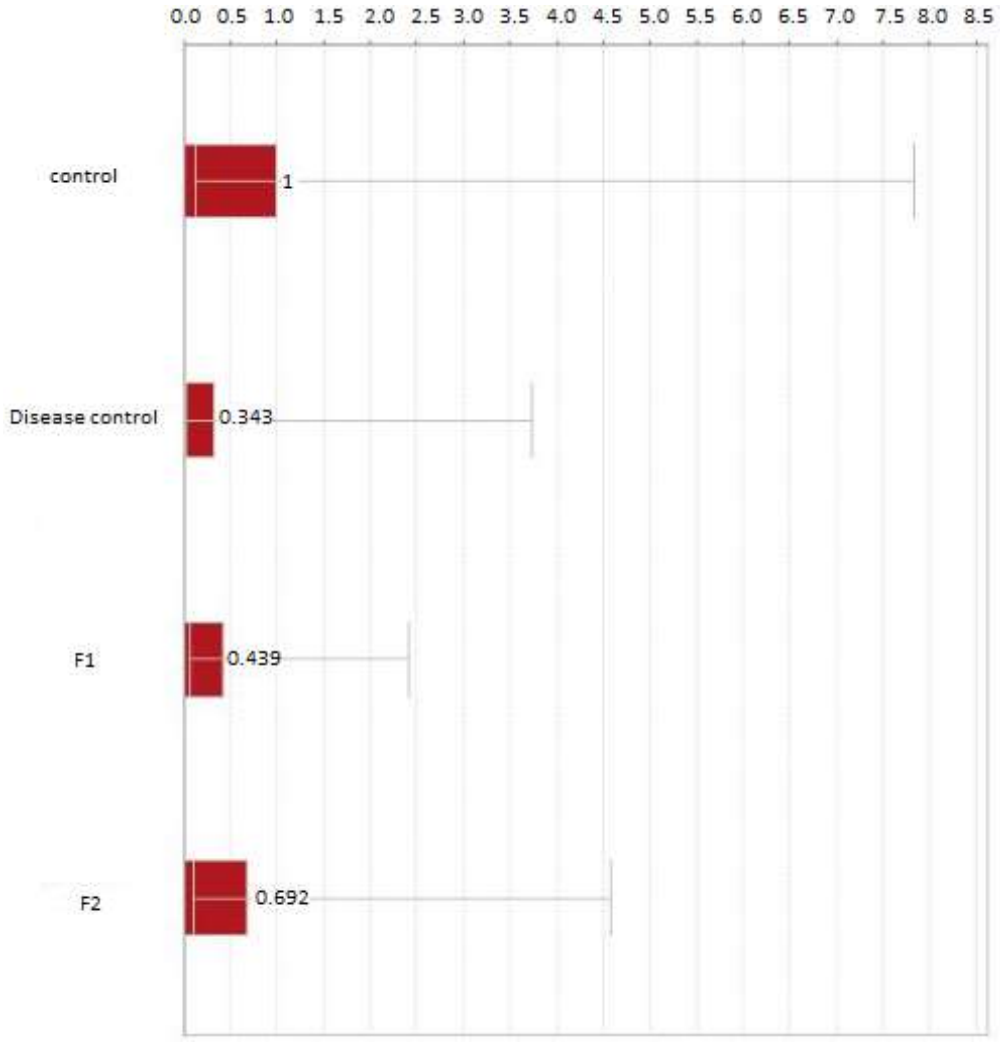

Figure 6. 23 Gene expression in mice treated with PBS, Interferon and final formulations

As shown in figure 6.23, 15 days treatment of interferon reduced the p11 mRNA level, almost by 66% which was increase by non-targeted nasal delivery of p11 lipoplex though not significant difference was found between nasal delivery and disease control. But the treatment with targeted lipoplex formulation (systemic route) significantly increase the mRNA level which was also visible in western blot studies. Increased mRNA level of p11 mRNA for systemic IGF-II targeted formulation significantly increase the protein level of p11 that ultimately helps in changed behavior and treating depression.

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