Chapter 6:

In vitro Evaluation

6.1 Introduction

Cell lines studies are very important as they provide method for preliminary assessment of direct effects on the tissues so as to establish clinical relevance in pathological states, screening and understanding of toxicity mechanisms. Basically, culturing cells implies that a tissue or outgrowth from primary explant is dispersed, either mechanically or enzymatically into a cell suspension which then is may form an adherent monolayer or a solid substrate or as a suspension in culture medium. The cells undergo proliferation in cultures forming a monolayer or suspension, which constitutes a passage. After several generations cell can transform into a continuous cell line having capacity of high growth and uniformity of population. Monolayer culture is the most common mode of culturing, in which cell are usually anchorage dependent and need a substrate for cell attachment before cell proliferation. In contrast anchorage independent cells (suspension cultures) are capable to proliferate without attachment, which is rare and is generally present in cells of hemopoeitic system.

In cell cultures, originally it was found that anchorage dependent cells attach to glass surface having negative charge. However, now they can also be cultured on plastics such as polystyrene which is surface treated with electric ion discharge or high energy ionizing radiations. Cells first secrete the extracellular matrix which binds the charged substrate and then cell binds to the matrix via specific receptors. There are different transmembrane proteins that are involved in cell-cell and cell-substrate interaction. The cell-cell interaction are brought about by class of self-interactive proteins i.e. homologous molecules in opposing cells interact with each other which are CAM (Calcium dependent) and Cadherins (calcium independent). On the other hand cell-substrate interactions are brought about by integrins which interact with matrix molecules. In addition, transmembrane glycoproteins are also known to be involved in cell adhesion.

The culturing protocol also requires disaggregation of attached monolayer culture using some proteases which can digest the extracellular matrix, thereby liberating the cell from matrix. Some cell types, such as epithelial are more resistant to disaggregation due to presence of tighter junctional complexes. Epithelial cells, if left for confluence for long, are hold tightly by desmosomes and tighter junctional complexes. Many of the adhesion molecules depend on Calcium ions, therefore, chelating agent such as EDTA are added to

trypsin solution during disaggregation. After cell attachment, the space between cells is filled with extracellular matrix (ECM), the composition being determined by the type of cell. The ECM, stays in dynamic equilibrium with cells and its complexity is significant contributor to phenotypic expression of cells attached to it. The components of ECM include collagen, laminin, fibronectin, hyaluronan, proteoglycans, and bound growth factors or cytokines.

Cell motility is also commonly evident in cell culturing experiments. They are capable of movement on substrate especially when the cell density is very low. The motile cells can be recognized by the presence of polarity, as a result of polymerization of lammepodium due to polymerization of actin in the direction of movement and adheres to the substrate. Subsequently the plasma membrane at opposite retracts and cell moves. The cells migrate in erratic manner, however once attaining confluence the migration stops under the influence of contact inhibition. Consequently cells form patches and the whole patch may also show signs of movement.

The cell cycle is divided into four phases: G1, S, G2, and M. The G1 phase decides the cell cycle progression at a number of restriction sites. The signals from environment regulate the entry into cell cycle. The free edges around the cell at low density under the influence of mitogenic growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGFs), or platelet-derived growth factor (PDGF) after their interaction with cell surface receptors. High cell density inhibits the cell proliferation after cell contact through induction of change in shape.

Primary cell cultures are often unsuitable for studies due to their instability i.e. they undergo continuous adaptive modifications and it is difficult to choose a period when entire cell population is homogenous or stable. After confluence some cells may transform and become insensitive to contact inhibition and overgrow, therefore it is necessary to keep the cell density low to maintain the original phenotype. After first subculture or a passage, the culture is called cell line. In each subsequent subculture a population of cell having capacity to rapidly grow will predominate while slow growing cells dilute out. In most cases culture becomes stable after three passage. The normal cells divide for a limited number of times due to progressive shortening of telomeres as these terminal sequences of DNA are unable to replicate in each cell division. However, germ cells, stem cells and

transformed cells express the telomerase enzyme which is capable replicating terminal sequences of DNA in telomeres.

The propagation of cell line also requires culture media preferably with defined chemical composition to ensure consistent quality and reproducibility. The physicochemical properties are taken into account to suit the requirements of purpose. Most of the cells grow well at pH 7.4. The carbon dioxide from the gas phase dissolves into medium to establish equilibrium with HCO_3^- ions to maintain the pH. Thus atmospheric CO_2 tension regulates the pH and standard equivalent HCO_3^- concentrations for different CO_2 have been provided in literature. Besides HCO_3^- other ingredients such as pyruvate, high concentration of amino acids are used as buffering agent in media. The cells also require oxygen, the depth of static culture should be kept within the range of 2-5 mm so as to maintain the rate of oxygen diffusion to the cells. The requirements of temperature depend upon body temperature of animal from which cells were obtained and is therefore kept at 37°C. They can tolerate a considerable drop i.e. can survive at 4°C for several days and can be frozen at −196°C, however; they cannot tolerate more than 2 °C above 37°C for more than few hours and will die quickly at 40°C and above. Therefore, consistency of temperature is required to obtain reproducible results.

6.1.1 Cell Culture

CFBE41o- cells were kind gift from Dr. Dieter Gruenert (California Pacific Medical Center Research Institute and Department of Laboratory Medicine, University of California, San Francisco CA). The protocol for culturing cells involved culturing in Eagle's minimum essential medium with Earls salt (EMEM) (Himedia, Mumbai) containing 2mM L-Glutamine, 10% fetal bovine serum (FBS) and 1% antibiotic antimitotic solution. Cells were maintained in a humidified incubator maintained at 5% CO2 at 37°C ± 2°C.

6.1.2 Culturing Protocol

The CFBE 41o- cells were maintained as monolayer in T-25 cell culture flasks, and medium was replaced two times in a week. After attaining confluency cells were sub-cultured in EMEM (Himedia, Mumbai, India) containing 10% FBS, 1% antibiotic antimitotic solution, 2mM L-Glutamine. The culture media was routinely examined during experimentation for freedom from deterioration and contamination. The decision to

subculture was taken based on confluency, multi-layering, evidence of mitosis etc. Following sub-culturing protocol was used:

1. The culture flask was taken to the sterile area and media was removed
2. The cells were washed with sterile phosphate buffer saline to remove the traces of serum which may inhibit the action of trypsin.
3. Trypsin-EDTA solution (2 ml) was added to the sides of the flask opposite to the cells and then turned down to ensure surface covering of the monolayer and left for 15-30 sec.
4. All but few drops of trypsin were withdrawn before complete dislodgment and flask was incubated at 37°C until the cells start to round up (5 mins).
5. Complete medium (2 ml) was added to disperse the cells dispersion with pipetting as continuous cell line requires vigorous pipetting for complete disaggregation.
6. Cells count was performed on haemocytometer.
7. Then appropriate seeding concentration was added to the flask and 10 mL of complete medium was added to it.
8. The flask was closed and incubated at 37 °C, 5% CO_2.

Each sub-culture represents one passage while the generation number was based on the split ratio. If the cells are growing correctly they reach the same concentration after same time in each cycle, if the seeding cell concentration remains constant. The standardization of culture condition was performed to maintain the phenotype stability which was achieved through regular maintenance and adherence to strict defined condition. The media was purchased from Himedia, Mumbai, India during all experimentation. The serum is also a potential source of variation, therefore; a single batch was used through experimentation.

6.1.3 Cell Counting using haemocytometer

1. Mix the trypsinized cell suspension thoroughly so as to disperse the cell into individual.
2. The Haemocytometer was cleaned using 70% ethanol
3. A cleaned coverslip was kept over the semi-silvered surface. Newton's ring formation was observed to ensure proper attachment of coverslip and correct depth of the chamber.

4. A 50 µl sample was taken using pipette and mixed with equal volume of trypan blue i.e. an exclusion dye for staining purpose to identify the dead cells. (The live cells exclude the water soluble dye due to their impermeable lipoid membrane)
5. After staining a sample was taken after dispersing cells and immediately transferred to edge of the haemocytometer chamber.
6. Care was taken not to overfill the chamber as it may change the dimensions of the chamber.
7. The second chamber was also filled in the similar manner.
8. Using the 10X objective the grid lines over the 16 corner square were focused.
9. The number of cells in the square was counted. Dead cells stained blue by trypan blue were excluded. Standard rule was followed not to count the same cell twice i.e. for each square the cells on top and left line were counted.
10. Viable cell count was performed on the on all 4 sets of 16 squares at each corner of Haemocytometer.
11. Number of cells in one set of 16 corner squares is equivalent to the number of cells in that set x 10^{-4} mL.
12. Average number of cells was calculated using following equation.

The total count from 4 sets of 16 corners = Average no. of cells/mL x 10^4 x 2. Where, 10^4 is conversion factor (conversion of 0.1 mm^3 to ml) and 2 is dilution factor.

6.2 Experimental

6.2.1 Cytotoxicity of polyplexes

Cell membrane destabilization before cellular internalization or chromosomal DNA interaction after cellular internalization could be the cause behind the cytotoxicity of free PEI [1]. Common method for determining cell viability such as dye exclusion (trypan blue) can be used. However 3-(4, 5-dimethylthiazole-2-yl)-2, 5-di-phenyl tetrazolium bromide (MTT) assay is rapid and versatile method [2].

The polymers as such were evaluated for cytotoxicity during initial screening (section 4.3.8.3 for PEI based polymers and 5.3.2.5 for chitosan based polymers). Now the polyplexes finally optimized on siRNA binding and stability challenge studies were subjected to cytotoxicity evaluation at increasing siRNA dose of 50 nM to 200 nM. The

CFBE41o- cells were seeded at density of 5000 cells/well in a 96 well microtiter plate (corning, New York) using EMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotic, 2mM L-Glutamine. [9]. The culture were grown for 24hrs in incubator maintained at 37 °C temperature and 5% CO_2 concentration and humidified with saturated CuSO4 solution. After 24hr the cells were exposed to different formulation in EMEM for 6h. Naked siRNA was also included to exclude the interference of therapeutic siRNA on viability of cells. After exposure period the media was replaced with complete medium containing 10% FBS, 2 mM L-Glutamine and 1% antibiotic. After 24 h, cells were washed with PBS and 20 ul of MTT reagent (5mg/mL) (Himedia, Mumbai) solution was added to each well. The reagent was allowed to react for 4 h under incubator condition, after which the medium in each plate was replaced with 100 ul of Dimethyl Sulfoxide (Himedia, Mumbai) and plate was shaken to dissolve the formazan crystals. The color of the dissolved formazan was measured using microtiter plate at 570 nm on a microtiter plate reader (Biorad, California). Cells treated with EMEM and 0.2% Triton X were used as negative and positive control. The absorbance values of cells treated with EMEM were taken as 100% cell viability and all other treatments were expressed relative to it.

6.2.2 Cell uptake

In order to perform cell uptake studies FAM labelled negative control siRNA (FAM-NC-siRNA) was used. The lyophilized stock supplied was reconstituted with NFW in amber coloured tubes and used for experiments. For quantitative measurements flow-cytometry was used while qualitative evaluation was conducted using confocal microscopy.

6.2.2.1 Confocal microscopy

The laser scanning confocal microscope (LSCM) is an essential component of modern day biomedical research applications. In a conventional microscopy the entire specimen is illuminated from a mercury of xenon source. However, in confocal microscopy the illumination is achieved by scanning one or more laser beams across the specimen to create an optical section of specimen in a non-invasive manner. It uses confocal pinholes that allow light coming only from the plane of focus to reach the photomultiplier tube detector and excludes the 'out of focus' light coming to the detector. This enables imaging of the living specimens and generation of 3-dimensional data in the form of Z-stacks. It uses laser

as light source, a sensitive photomultiplier tube detector and a computer to control the scanning mirrors and build images.

The optical path used in confocal microscopy is based on conventional reflected light wide-filed epi-fluorescence microscope with a point light source and a pinhole in front of detector which are confocal with each other. The specimens are labeled with one or more fluorescent probes. The confocal microscopy also offers the advantage greater resolution due to use of highly sensitive photomultiplier tube detectors. The series of time-lapse run can be converted into a 3-D image from the obtained data with time as the z-axis. This can be useful for observing physiologic changes during development. Further a 4-dimension data set can be produced consisting of three spatial dimensions X, Y, Z and time as fourth dimension. In cellular biology confocal microscopy has been used for visualizing intracellular organelles, cellular uptake, intracellular localization of drugs and drug delivery systems using fluorescent probes.

Protocol: Cells were seeded at a density of 10^4 cells/well in a 24 well plate containing 0.17 mm thick flame sterilized cover glass and were allowed to grow for 24 h in EMEM at 37 °C temperature and 5% CO_2. After 24 h cells the media was removed and cells were washed with sterile PBS. Then the cells were exposed to formulations containing FAM labeled negative control siRNA (FAM-NC-siRNA) at siRNA concentration of 100 nM. After 6 h of exposure cells were washed with PBS for twice to ensure removal of residual formulation. Then the cells were fixed with 4% paraformaldehyde solution (1 mL/well) and incubated at room temperature for 3-5 min. The paraformaldehyde was immediately removed after exposure time and cells were washed with PBS three times accompanied by intermittent shaking for each wash to remove the traces of paraformaldehyde. Then the nuclei of the cells was stained with 4',6-diamidino-2-phenylindole (DAPI) at 1 ug/mL concentration with enough volume to cover the cells and kept for 15 min at room temperature for dye permeation under protection by aluminum foil. Then cells were washed once with PBS. The coverslips were mounted on glass slide using PBS:glycerin solution (50:50 and confocal microscopy was performed using confocal laser scanning microscope. The negative control for the experiment was Naked FAM-NC-siRNA and positive control was Lipofectamine 2000 (L2K) complexed siRNA.

Table 6.1: Cell line treatment parameters for confocal microscopy and flow cytometry of LPEI based formulations

Sr No	Formulations	Cells	Treatment	Condition
1.	Naked siRNA	CFBE 41o- cells	100 nM FAM-NC siRNA	Incubation time=6 h Temperature = 37°C (5% CO_2)
2.	HELPEI-35		100 nM FAM-NC siRNA	
3.	HELPEI-45		100 nM FAM-NC siRNA	
4.	LPEI		100 nM FAM-NC siRNA	
5.	L2K		100 nM FAM-NC siRNA	

Table 6.2: Cell line treatment parameters for confocal microscopy and flow cytometry for chitosan based formulations

Sr No	Formulations	Cells	Treatment	Condition
1.	Naked siRNA	CFBE 41o- cells	100 nM FAM-NC siRNA	Incubation time=6 h Temperature = 37°C (5% CO_2)
2.	LMWC-29		100 nM FAM-NC siRNA	
3.	LMWC-29-PS-12		100 nM FAM-NC siRNA	
4.	MMWC		100 nM FAM-NC siRNA	
5.	PS			
6.	L2K		100 nM FAM-NC siRNA	

6.2.2.2 FACS

Flow-cytometry is a powerful technique for characterizing cells in clinical diagnosis and biomedical research for quantifying aspects about their size, internal complexity and surface markers. In a flow cytometer the suspension of cells is hydro-dynamically focused in a single cell wide stream of fluid containing a fast moving sheath fluid around the slow moving cell suspension emerging through a 70 um nozzle. This is achieved by with air or gas pressure and the differential pressure between the streams controls the samples introduction rate. This laminar steam stream of particles is subsequently interrogated by one or more laser beams placed perpendicular to it and only illuminate single cell at a time. At this point the laser is scattered at the same wavelength at different directions. The light scattered in forward direction (FSC) is proportional to the size of the cells. While the light scattered in perpendicular direction (SSC) correlates with intracellular granularity or complexity. Thus scattering itself gives information about the size and composition of the cells [3].

The second technique of detection relies on use of fluorescent probes attached to cells, which fluorescence after interaction with laser at interrogation point and emit light at longer wavelengths. Here, the non-fluorescent cells will be counted as negative while the fluorescent cells will be called as positive cells. Further, the intensity of emission gives information about the number of fluorescent probes. Downstream the interrogation points, the particle stream is broken into discrete droplets which can be selectively charged and deflected using an electric field into a collector and the remainder are disposed. The results of fluorescence and scatter are displayed as histogram.

Before starting the experiment appropriate controls are needed to enable interpretation of the results in the context of the purpose of experiment. At least three controls are essential in any experiment which are: set up control (instrument), specificity control (gating) and biological comparison control [4]. 'Setup controls' are required to ensure that instrument is properly set up with respect to photomultiplier voltage gains and compensation; 'Specificity or gating controls' are used to set location of gates or graphical regions to classify the cells as required for the purpose of the experiment. A 'biological comparison control', consisting of unstained/unstimulated cells in a biologically relevant conditions, is required to set up positive/negative boundaries

Protocol: For FACS analysis cells were seeded in 24 well plate at cell density of 5 X 10^5 cells/well. The cells were allowed to grow for 24 h in EMEM. After 24 h the cells were treated with formulations containing FAM-NC-siRNA at a concentration of 100 nM and kept for 6 h in incubator maintained at 37°C with 5% CO_2 in a humidified conditions. During this period the cells were supposed to internalize the formulations depending on the transfection efficiency. After incubation the cells were washed three times with cold PBS pH 7.4 to remove the residual formulations and harvested using trypsin to obtain a cell suspension in PBS pH 7.4. Before analysis the cell suspension was passed through 70 µm cell strainer to disperse cell aggregates, if any and analyzed for % cell uptake using fluorescence activated cell sorter (FACS BD, USA). The positive control was Lipofectamine 2000 (L2K) complexed siRNA.

6.2.3 Gene knockdown efficiency

RT-PCR is a powerful tool for the detection and quantification of mRNA. It is popular because of high sensitivity, good reproducibility, and wide dynamic quantification range [5]. It allows researchers to amplify specific pieces of DNA more than a billion-fold [6]. In PCR a thermostable polymerase synthesizes a complementary sequence of bases to any single strand of DNA containing a double stranded starting point. The starting points can be chosen by user corresponding to gene of interest and they are known as primers. During PCR the temperature cycling is used to control the activity of thermostable polymerase and binding of primers. At the beginning the temperature is kept at 95^{o}C where all double stranded DNA will melt. Then temperature is reduced to ~60^{o}C, depending on primer, to allow the primer to bind the target gene. The polymerase subsequently binds the double stranded DNA and starts copying. This temperature when repeated several times leads to exponential increase in copies of target DNA sequence. The amplified gene can be observed by running on agarose gel and staining. However, in conventional PC the gel based analysis cannot give time dependent quantity curve.

In Real Time PCR this process is monitored in real time using fluorescent probes of double stranded DNA and detecting them with a camera. The RT-PCR offers advantages such as: a direct look into the reaction, precise calculation of efficiency of reaction, eliminating the need to run gels and performing a truly quantitative analysis of gene expression rather than semi-quantitative as in normal PCR [7]. The RT-PCR uses asymmetric cyanine dyes e.g. SYBR green I and BEBO, which do not interfere with polymerase chain reaction. The primer-dimer formation can be easily identified from melt curve analysis. The primer design is also a crucial aspect of RT-PCR. It depends on choice of amplicon as well. The amplicon is generally kept to < 300 base pairs SYBR green based detection while 50-150 base pairs for probe based detection. The primers are generally 15 – 20 base pair and contain 20-80% CG units.

The quantification using PCR can be standard/absolute or relative. For absolute quantification a standard curve of Ct vs log (conc. Of standard gene) is required to be generated. While in relative quantification a mathematical equations are used to calculate

expression level relative to non-treated sample. Further it is normalized to a housekeeping gene which is expressed at constant level and has same amplification efficiency. The Ct values are used for quantification purpose which are fractional PCR cycle number at which the reporter fluorescence level is greater than minimal detection level. This ensures increased accuracy and reproducibility, since; the difference in fluorescence of end product and blank cannot be used for quantification as fluorescence cease to increase proportionately at the end of reaction as the dye to base binding ratio decreases over the course of reaction. The Ct values are arbitrarily chosen by the software based on the standard deviation of the baseline and it is generally kept 10 times the SD of baseline signal from cycle 3 to 15.

Protocol: CFBE 41_{O}- cells were seeded on a 24 well plate at a density of 10^5 cells/well were incubated for 24 hr to get approximately 80% confluency. After incubation, cells were treated with ENaC siRNA formulations at three different siRNA concentrations i.e. 25 nM, 50 nM and 100 nM. Untreated cells were used to determine the basal gene expression level against which gene expression levels of formulations were determined. Cells transfected with L2K were used as positive control according to manufacturer's protocol. After incubation for 48 hr, total RNA was isolated using TRIzol reagent and reverse transcription into cDNA was carried out using RNA to cDNA conversion kit. mRNA level was quantified using Step One real time PCR using SYBR Green Mastermix, forward and reverse primers and 2 ng of cDNA in a total volume of 20 µL. The mRNA expression level of ENaCα gene was normalized against housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reaction protocol and specification followed are described below:

6.2.3.1 Selection of primers

Primer design tool of NCBI (National Center for Biotechnology Information) was referred for primer selection (Table 6.3). The primers for human ENaC siRNA were 5′-CCTGGAATCAACAACGGTCT-3′ for forward and 5′-AGGGTTTCCTTCCTCATGCT-3′ for reverse which will form a PCR product 188 bp. Primers for housekeeping gene GAPDH were 5′-ATCCCATCACCATCTTCCAGGA-3′ for forward and 5′-CAAATGAGCCCCAGCCTTCT-3′ for reverse which will form a PCR product of 122 bp.

6.2.3.2 Total RNA isolation

The gene expression analysis also depends on integrity of isolated RNA, therefore isolation of intact total RNA is primary requirement for gene quantification. The absolute quantification that normalize specific mRNA expression against total RNA (g/g of total RNA). The long mRNA are prone to degradation by RNase during tissue sampling, RNA purification and RNA storage. In addition to the cellular RNase there are several RNases that are present in environment. The RNA samples may get contaminated by DNA and even minor quantities can get amplified in PCR. Therefore, a properly optimized laboratory procedure was used for RNA extraction:

1. One mL of TRIzol reagent (1 mL/10cm^2) was added to each well and allowed to incubate for 5 min at room temperature.
2. The sample was transferred to 2ml of autoclaved eppendorf (DEPC treated, RNase free). Then 200µl of chloroform was added and mixed vigorously for 15 sec and incubated at room temperature for 2-3 min.
3. The machine was pre-maintained and samples were centrifuged at 12000g for 15 min at 2-8^{0}C
4. Then 50% of the aqueous phase, above the fairly visible interphase, was transferred to fresh tubes. The aqueous phase contains both RNA and DNA, however, RNA, being of smaller fragments, resides in the top of the aqueous phase.
5. To the aqueous phase 0.5 mL of isopropyl alcohol was added and incubated at for 10 min at room temperature. Further, sample was incubated at -20^{o}C to precipitate the RNA.
6. The sample was centrifuged at 12000g for 10min at 2-8^{0}C to obtain the RNA pellet.
7. The supernatant was removed and 75% ethanol was added to wash the pellet by mixing with vortex again centrifuged at 7500g for 5min at 2-8^{0}C
8. The supernatant was removed and pellet was allowed to semi air dry.
9. The washed pellet was dissolved in 50µl DEPC treated water by incubation at 55-60^{0}C for 10min.
10. The RNA was checked on 1.2% agarose by loading 2 ul of sample with loading dye.

11. The RNA concentration was checked by OD (1 O.D = 33 µg/ml) using nanodrop and the purity was assessed from A260/A280 ratio which was between 1.8-2.1

Table 6.3 Details of primers

Primer	Sequence (5'->3')	Template strand	Length	Tm	GC %
Human ENaCα					
Forward primer	CCTGGAATCAACAACGGTCT	Plus	20	58.4	50
Reverse primer	AGGGTTTCCTTCCTCATGCT	Minus	20	59.2	50
GAPDH primers					
Forward primer	ATCCCATCACCATCTTCCAGG	Plus	21	59.8	52.4
Reverse primer	CAAATGAGCCCCAGCCTTCT	Minus	20	60.9	55.0

6.2.3.3 RNA to cDNA conversion

RNAs are highly unstable and sensitive, and are prone to degrade by the RNases. On the other hand DNA is fairly stable, therefore; RNA is converted to cDNA to store the information in RNA in a stable form. The RNA to DNA conversion is brought about by RNA-dependent DNA polymerase, known as reverse transcriptase. Using RNA as template it can produce cDNA. It also needs a primer with a free 3′-hydroxyl group. In eukaryotic mRNAs, a poly-A tail is present at their 3′-ends, therefore; a poly-T oligonucleotide can be used as a primer. During reaction the primer gets annealed to the 3′-end of the mRNA. 3′-end of the primer is extended by the reverse transcriptase producing a RNA-DNA hybrid molecule. Finally using RNase H or alkaline hydrolysis, the RNA strand of this RNA-DNA hybrid molecule is digested. The following step wise protocol was used for cDNA synthesis:

1. To convert RNA to cDNA the high capacity RNA-to-cDNA Conversion Kit was utilized.
2. Kit components were removed from their storage conditions and allowed to thaw on ice.
3. 1.5 microgram of RNA /20 µL of reaction was used for conversion.
4. The Reaction set up used is given in Table 6.4.

Table 6.4 RNA to cDNA conversion parameters

Component	Volume/ Reaction
Sample	9
2× RT (reverse transcription) Buffer	10
20× RT (reverse transcription) Enzyme	1

5. To the each well of 48 well plate, 20 µL of RT (reverse transcription) reaction mix was added for real time PCR reaction.
6. Plate was sealed with sealer and centrifuged to spin down the contents and to remove air bubbles. Plate was placed in the sample holder of PCR system and following cycle given in Table 6.5 was run:

Table 6.5 Steps of PCR cycle

Parameters	**Step 1**	**Step 2**	**Step 3**
Temperature (^{o}C)	45	95	4
Time (min)	30	10	Storage

6.2.3.4 Real Time PCR

Once the cDNA was obtained from mRNA. The quantification of gene expression was performed on RT-PCR using SYBR green based detection and gene knock-down was accessed with respect to the control. The reaction was set as per below composition (Table 6.6):

Table 6.6 mRNA quantification – reaction parameters

Component	**Volume/ Reaction (µL)**
Forward primer	0.7
Reverse primer	0.7
cDNA	1.5
Master Mix	7.5
Nuclease-free H_2O	q.s. to 15

To the each well of 48 well plate 15 µL of RT reaction mix was added for real time PCR reaction. Plate was sealed with sealer film and centrifuged to settle down the contents

and to remove any air bubble. Plate was placed in the sample holder of RT-PCR machine and following cycle (Table 6.7) was run:

Table 6.7 RT-PCR cycle steps refer the stages below for details

Parameters	Step 1	Step 2	No. of cycles
Temperature (°C)	95	60	45
Time (seconds)	15	60	

6.3 Result and Discussion

6.3.1 PEI based siRNA delivery Systems

6.3.1.1 Cytotoxicity of polyplex

Cytotoxicity was assessed at increasing dose of siRNA polyplexes prepared at the optimized n/p ratio. The naked siRNA was used to see the effects of therapeutic siRNA on viability of cells. The cytotoxicity results of polyplex showed that, at the optimized composition they were non-toxic in delivering increasing siRNA dose. It was observed that there was no significant difference in % cell viability for naked siRNA, HELPEI-35, HELPEI-45 formulations at increasing dose of siRNA upto the highest dose of 200 nM when compared with untreated control ($p > 0.05$). However, LPEI showed significant toxicity at concentration of 150 and 200 nM ($p < 0.05$).

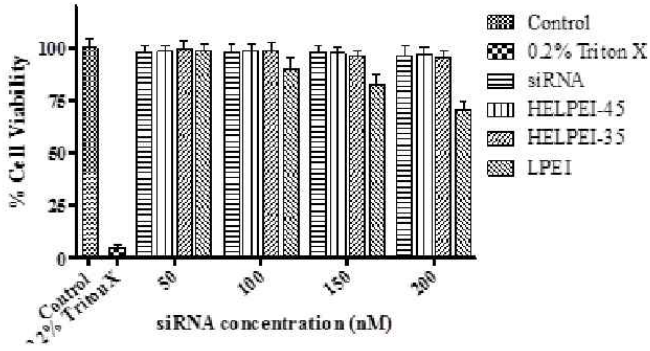

Fig. 6.1: Comparison of cytotoxicity of siRNA polyplexes

6.3.1.2 Confocal microscopy

The results of confocal microscopy showed the cell uptake of naked siRNA and different polyplex prepared from LPEI and HELPEIs and that of commercial transfecting agent,

L2K. As seen from the Fig. 6.2A, naked siRNA showed negligible cellular uptake, while polyplexes showed marked cellular uptake. The observed low uptake of naked siRNA is direct result of high molecular weight as well as high hydrophilicity which interferes with cell membrane interaction and subsequent cell uptake. There are also chances that the naked siRNA may get degraded by hydrolysis or by nucleases. L2K was also included for the comparison purpose and also to ensure suitability of the experimental procedures. The commercial transfecting agent also showed considerable cell uptake as shown in Fig. 6.2E.

On the other hand, LPEI, being cationic charged, forms complex with siRNA and protects it from the degradation. The formed complex is condensed in nature and is of small size. Further, due to residual positive charge, as observed in zeta potential measurements, helps it to interact with the negatively charged cell membrane which is subsequently internalized through endocytosis. The fluorescence of this type of endocytosed FAM-NC-siRNA can be visualized in confocal microscopic images. However, LPEIs association with the cells had a typical pattern, in which small particles were observed bound to the cell membrane high-lighting the periphery of the cell. The cell association was also in the form of few large aggregates (Fig. 6.2B). The literature reports also shows similar observations in case of LPEIs [8, 9].

The HELPEI-35 and HELPEI-45 showed higher cytoplasmic uptake compared to both naked siRNA and LPEI. In contrast to LPEI, the fluorescence in confocal images of both HELPEIs was throughout the cytoplasm rather than preferentially at the cell periphery, which is a good indicator of cytoplasmic intake of polyplexes (Fig. 6.2C-D). The zeta potential values had indicateed positive surface charge (> +17 mV) required for cell membrane interaction. The long chain PEGylated carriers have been reported to interfere with cell interaction through charge masking and large hydration shell leading to lower cell uptake [10, 11]. However, the small length hydrophilic functional groups present were smaller than PEG to interfere with cell surface interaction. At the same time the small length hydrophilic surface was efficient at reducing cell damage due to positive charge as evident from the erythrocyte aggregation and haemolysis studies.

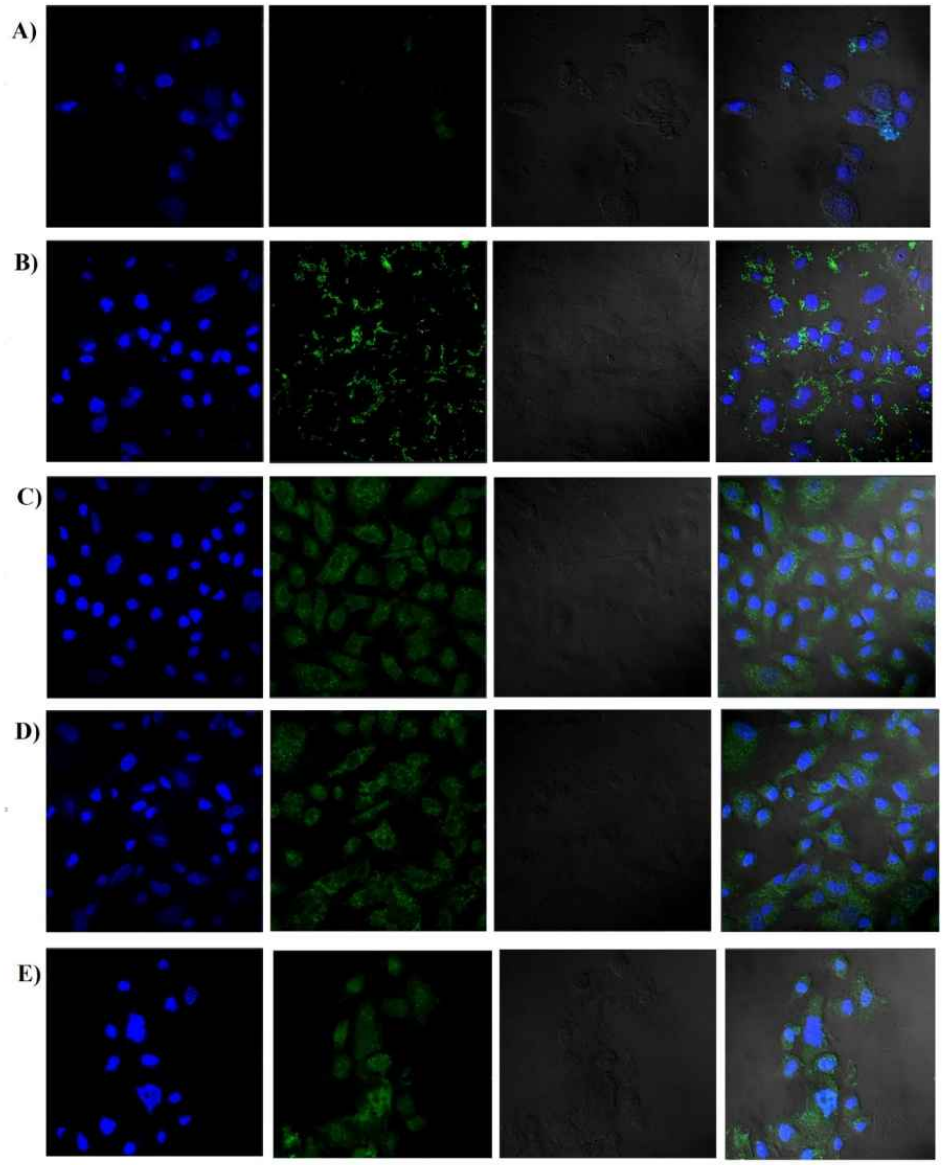

Fig. 6.2: Cell uptake of screened formulations by confocal microscopy; A) Naked siRNA, B) LPEI, C) HELPEI-35, D) HELPEI-45, E) L2K

6.3.1.3 FACS

After evaluation for cell uptake the formulations were subjected to flow cytometry analysis using FAM-NC-siRNA at 100 nM concentration for all the formulations to get quantitative details of cell uptake. Fig. 6.3, show 2D histograms as well as dot plots of FACS analysis of cellular uptake of naked siRNA and polyplex formulations, the results of which are also tabulated in Table 6.8.

Table 6.8: Uptake of various formulations in CFBE 41o- cells

Formulations	% cell uptake
Naked siRNA	9.07±1.41
LPEI	62.75±1.84
HELPEI-35	80.16±2.88
HELPEI-45	74.12±3.61
L2K	67.23±2.58

It was apparent from the data that, cells treated with naked FAM-NC-siRNA and showed very low level of uptake (~9.0%), while PEI based positively charge polyplexes and commercial transfection standard, L2K, showed higher uptake (>60%) inside cells. The developed highly biocompatible modified LPEI, i.e. HELPEI-35 and HELPEI-45 showed a positive improvement in cell uptake after substitution compared to that of LPEI. The results of FACS corroborate the qualitative observations of confocal microscopy. Thus based on FACS data the order of cell uptake could be stated as:

HELPEI-35 > HEPEI-45 > L2K > LPEI > Naked siRNA

The L2K, owes its transfection efficiency to ease of interaction with cell membrane and subsequent internalization into the cell, which depend of milieu of variables such as particle size, zeta potential, hydrophilic lipophilic balance, colloidal stability of vector, molecular weight, toxicity of the polymer etc [12-14]. The experimental observations were in agreement with the literature reports [15, 16]. On the other hand siRNA is unable to interact with cell due to negative charge, large size, hydrophilic nature, which concomitantly results in lower cell uptake of ~ 9.0%.

In contrast, LPEI and HELPEI showed significant cell uptake compared to naked siRNA ($p < 0.05$). The LPEI due to its cationic charge and small size is able to show

significant cell uptake. The increase in DS was supposed to decrease the cationic charge in HELPEIs; however, the residual cationic charge even after decreased ionization was sufficient to encourage interaction with the cell membrane and allow endocytosis of the polyplex. However, the difference in cell uptake of HEPEIs and LPEI was significant ($p < 0.05$).

When comparing the cell uptake of HEPEI-35 and HELPEI-45, the cell uptake in case of HELPEI-35 was higher than that of HELPEI-45 ($p < 0.05$). This means that increase in substitution beyond certain point does not lead to significant benefit in the cell interaction and cell uptake as result of significant suppression of ionization of HELPEI-45.

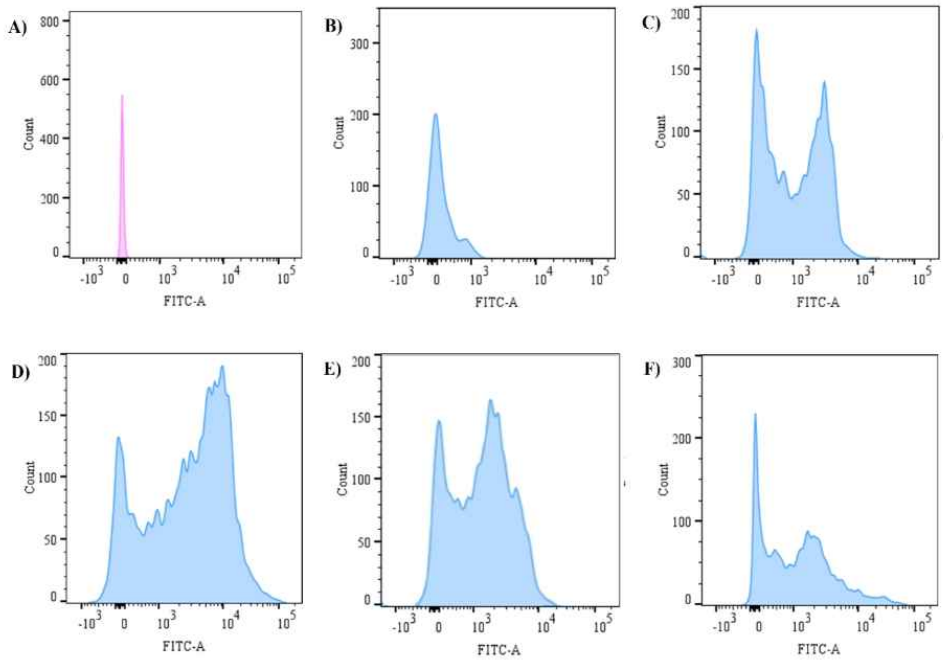

Fig. 6.3: Histogram plots from flow cytometer after cell uptake study A) unstained control, B) naked siRNA, C) LPEI polyplexes, D) HELPEI-35 polyplexes, E) HELPEI-45 polyplexes, F) L2K.

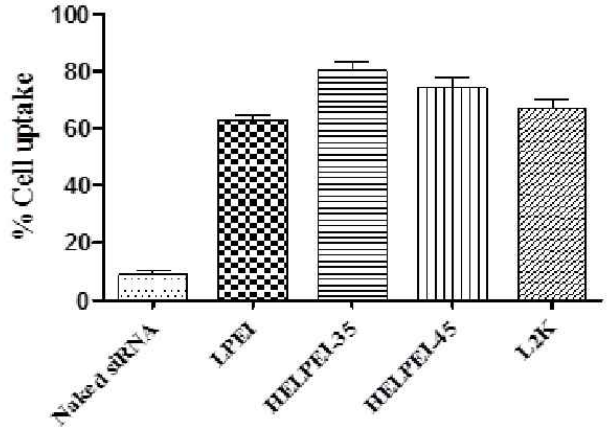

Fig. 6.4: % cell uptake observed in FACS study of prepared polyplexes

6.3.1.4 RT-PCR

To study the gene knockdown Real Time PCR was used. The high resolution melt curve (Fig. 6.5) of both the DNA amplicons showed sharp melt peaks for ENaC and GAPDH respectively indicating no primer dimer formation and a homogenous specific gene amplification product was generated.

The cells were transfected with siRNA concentration at 25 nM, 50 nM and 100 nM. Highest knockdown was observed at 100 nM siRNA. At this level, naked siRNA showed 89.03±2.38 % gene expression. At the same concentration HELPEI-35 and HELPEI-45 formulations showed gene knockdown of 33.32±2.39% and 30.54±2.14% respectively, which was significantly higher than LPEI ($p < 0.05$). The L2K used as commercial standard also showed good transfection efficiency (37.34±1.16 %), it was comparable to HELPEIs at all concentrations. To confirm that gene silencing was due to siRNA only and not due to any off-target effect or non-specific inhibitions, we also included polyplexes of HELPEI-35-NC-siRNA, which showed negligible gene knockdown compared to the control.

Although FACS studies had shown good cell uptake for LPEI, the transfection was not in concordance with it. This can explained by confocal studies which showed that cellular association LPEI was preferentially on the membrane with little cytoplasmic appearance. It might be possible that surface associated polyplex were not internalized or

unable to escape from endosomes after internalization. Thus, positive surface character may ensure cell attachment, the subsequent endocytosis and endosomal escape and unloading is also a limiting factor in gene expression. Similarly, cationic polymers such as poly-l-lysine, poly arginine etc. are less efficient in transfection because of their poor buffer capacity in the endosomal pH range [17].

The endosomal escape is essential, otherwise the nucleic acid is readily degraded following drop in pH in endosomal-lysosomal pathway. The superiority of HELPEIs over LPEI could be attributed to endosomal escape capacity, as apparent from buffer capacity measurements and greater cytoplasmic fluorescence in confocal images. Further, though HELPEI-35 and HELPEI-45 were equivalent in buffer capacity, the difference in gene expression between HELPEI-35 and HELPEI-45 can be explained based on results of FACS, which showed that HELPEI-35 was more efficient in cell uptake due to greater positive surface charge than HELPEI-45. Further, it can be argued that HELPEIs may also differ in nucleic acid unloading characteristic due to difference in charge density. The optimum binding affinity required depends on charge density and n/p ratio used.

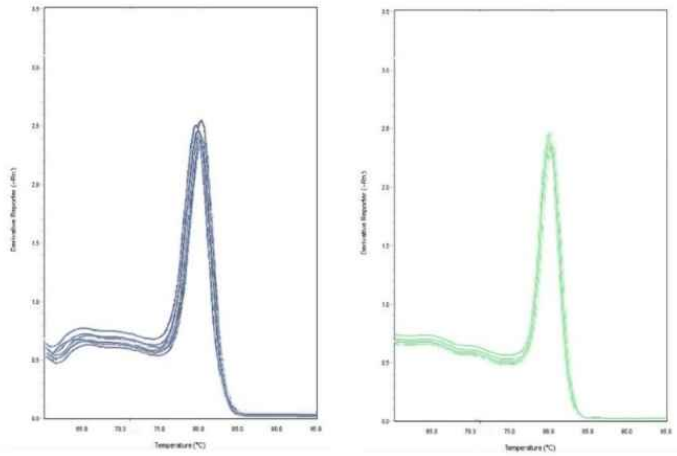

Fig. 6.5: Melt Curve for RT-PCR: ENaC (Blue color), GAPDH (Green color)

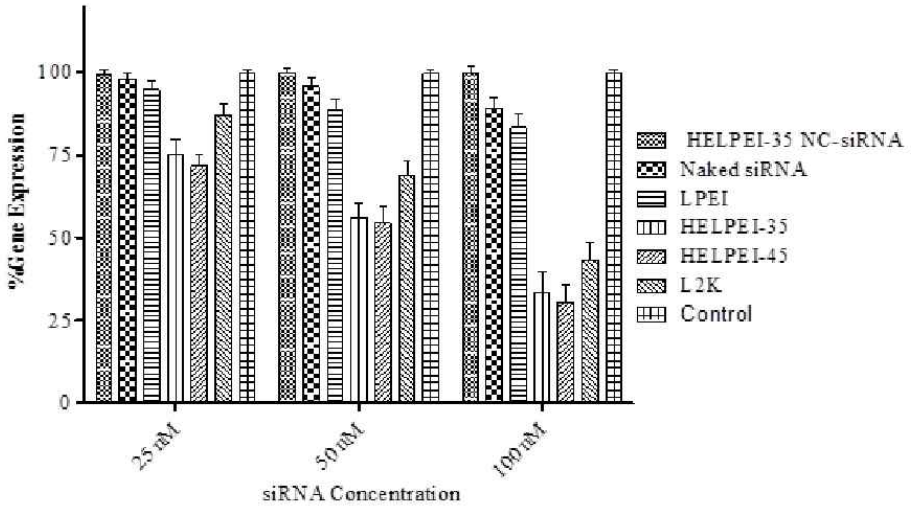

Fig. 6.6: % Gene expression in CFBE41o- cells after treatment LPEI based formulations

6.3.2 Chitosan based siRNA delivery system

6.3.2.1 Cytotoxicity of polymer

Fig. 6.7 shows the viability of CFBE 41o- cells after 24h incubation with different concentrations of siRNA polyplex. As observed in case of polymer the chitosan based polyplex vectors also showed negligible toxicity i.e. > 90% after 24h. A sequence of was used to exclude the interference of therapeutic siRNA on viability of cells. Only the PS alone was found to be toxic if used alone to deliver high doses of siRNA. However, the small quantities present in formulation have negligible effect on viability.

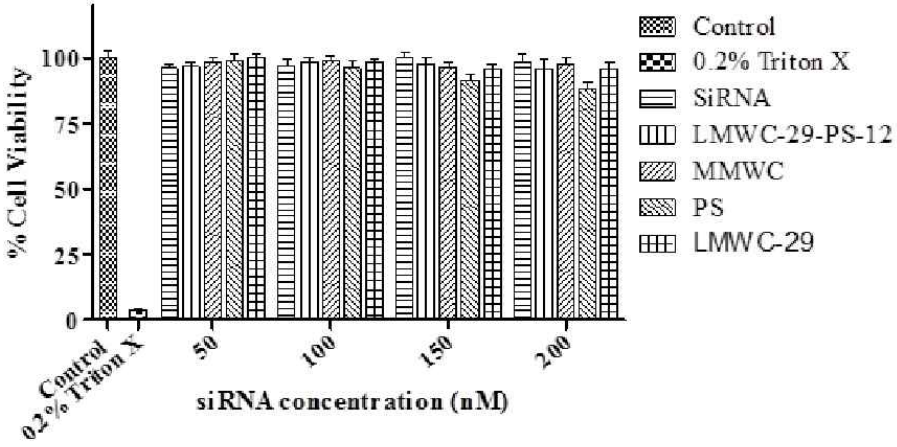

Fig. 6.7: MTT assay for cytotoxicity of chitosan based polyplexes

6.3.2.2 Confocal microscopy

The cells exposed to naked FAM-NC-siRNA showed negligible cell uptake neither the non-specific binding to cells. However, in case of MMWC, the fluorescence was observed around the cell membranes (Fig. 8B). It could be attributed to self-aggregation and precipitation in the culture conditions, as observed in solubility studies. Similarly, LMWC-29 showed less fluorescence in confocal images. In spite of improved colloidal stability, premature release of siRNA from weak complexes of LMWC-29 and the unionized nature at physiologic pH might be responsible for lower cell uptake. The stabilized LMWC-29-PS-12 polyplex showed significant cell uptake compared to MMWC and LMWC-29. LMWC-29-PS-12 were stable and had optimum size (143.7±4.56) for cell uptake. Further, the PS fraction confers positive surface charge (+12.8±1.38 mV) to the conjugate even at 7.4 pH, where uncomplexed free amino groups of chitosan would be unionized. PS was also used as a control to prove the significance of modification. It showed significant cell uptake, comparable to conjugate. It could be due to the formation of stable polyplex with siRNA and positive surface charge. L2K, as positive control, showed comparable uptake to that of LMWC-29-PS-12 conjugate.

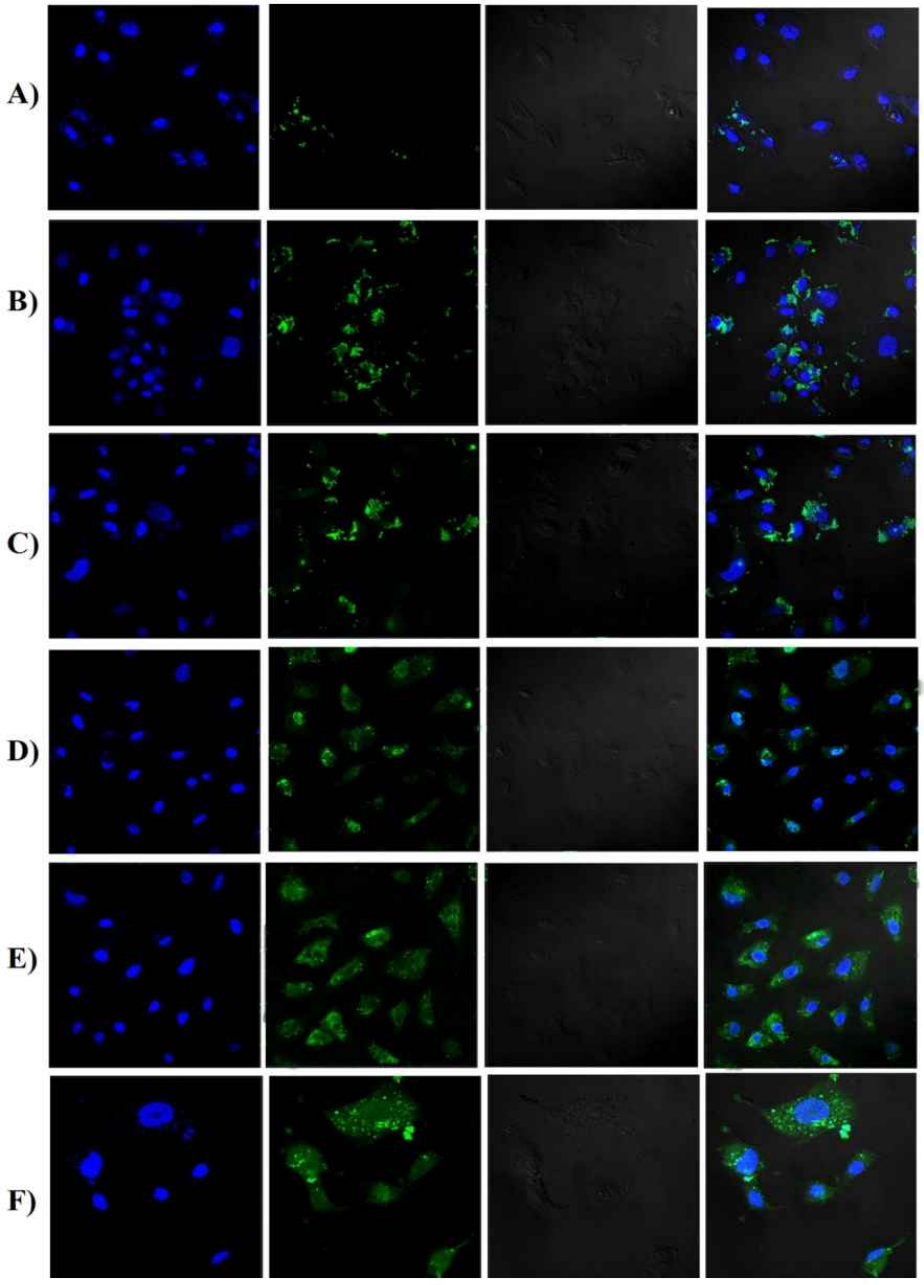

Fig. 6.8: Confocal microscpy images of A) FAM-NC-siRNA, B) MMWC, C) LMWC-29, D) PS, E) LMWC-29-PS-12, F) L2K

6.3.2.3 FACS

To more precisely understand the cell internalization event, we quantified the internalized FAM-NC-siRNA using FACS analysis. The results provided % cell uptake depending on the number of fluorescent cells. The results are shown in the form of histograms as follows:

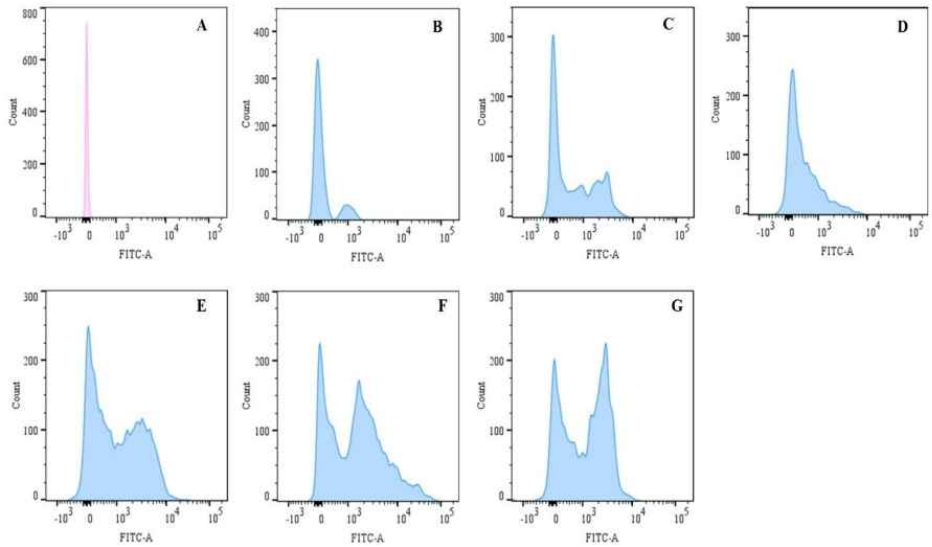

Fig. 6.9: Results of FACS analysis of different polyplexes A) Untreated cells B) Naked FAM-NC-siRNA, C) MMWC polyplexes, D) LMWC-29 polyplexes, E) PS polyplexes, F) LMWC-29-PS-12 polyplexes, G) L2K

To more precisely understand the cell internalization event, we quantified the internalized FAM-NC-siRNA using FACS analysis. Naked siRNA showed negligible levels of uptake (<14%) while formulations led to higher uptake inside cells. Both the MMWC and LMWC-29 showed low levels cell uptake of ~45% and 36% respectively. The LMWC-29 showed the least level of cell uptake, indicating that apart from physical stability the unionized nature of chitosan would be the limiting factor for low transfection with chitosan based systems.

In contrast, PS showed cell uptake (~63%) greater than the MMWC and LMWC-29. This could be due to stability of complex, small size and presence of positive surface charge were sufficient to interact with the cell and undergo internalization. Finally, among all the formulations TPP cross-linked LMWC-29-PS-12 polyplex**,** which had combined physicochemical attributes of LMWC and PS, showed cell uptake (~70%) comparable to

that of transfection standard, L2K (~68%). The results of FACS were in full agreement with the confocal laser scanning study.

Table 6.9: Quantitative cell uptake of various formulations

Formulations	% Cell uptake
Naked siRNA	13.90±1.69
MMWC	44.78±2.49
LMWC-29	35.88±1.76
PS	63.37±2.48
LMWC-29-PS-12	70.19±3.09
L2K	67.89±2.86

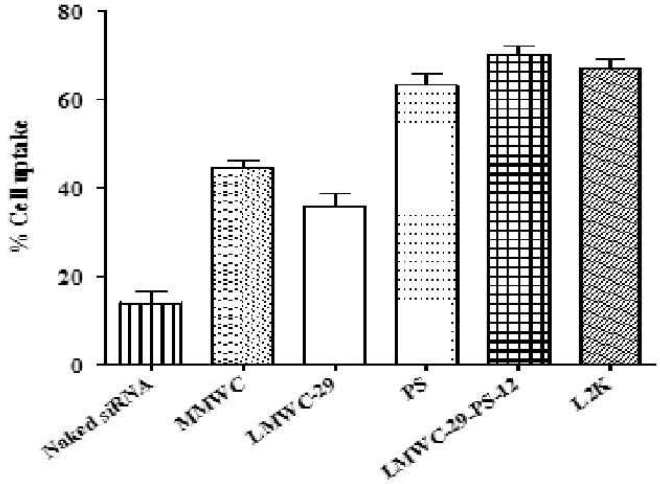

Fig. 6.10: % cell uptake observed in FACS

6.3.2.4 RT-PCR

Based on the results of cytotoxicity, confocal, FACS the formulations were subjected to RT-PCR analysis. The mRNA expression levels were estimated as % of ENaC-mRNA expression of control. The gene silencing could also occur due to non-specific effects of siRNA sequence. The NC-siRNA loaded polyplex did not cause knockdown of ENaC mRNA, which confirmed the sequence specificity of siRNA to inhibit ENaCα.

While comparing remaining formulations, at each siRNA concentration tested, the maximum activity was attained at highest concentration of siRNA treated. At the highest dose, naked siRNA reduced mRNA expression at very low extent (~14%) and commercial transfection standard L2K showed significant mRNA knockdown of 44.46 % at the highest dose. LMWC-29 polyplexes showed very less mRNA knockdown, which could be due to lack of cell interaction as observed in FACS studies. However in case of PS polyplexes, in spite of showing greater uptake in FACS studies, the subsequent gene expression was very less i.e. ~67% at the highest dose. This could be attributed to the lack of endosomal escape capacity in PS-siRNA complex, as observed in poly-L-lysine, poly-l-arginine based vectors.[18] The stabilized LMWC-29-PS-12 polyplexes showed significant mRNA knockdown of 42.63%**.** They were able to interact with the cell surface, due to presence of PS fraction, and undergo cell internalization through endocytosis. Further, subsequent to endocytosis, the associated LMWC fraction contributes to endosomal escape and cytosolic release of siRNA from the formulation.

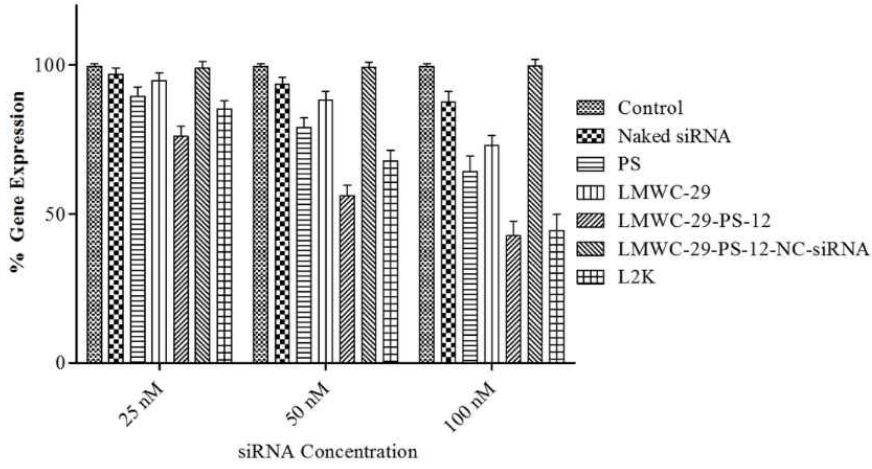

Fig. 6.11:% Gene expression in CFBE 41o- cells after treatment with various formulations

6.4 References

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