Chapter 3:

Analytical Methods

3.1. Selection of siRNA

The low level of observed gene correction through CFTR gene delivery is inefficient to normalize Cl- flux as well as Na^+ hyperabsorption. The ENaC activity is fundamental mediator of CF pathogenesis and must be normalized to get complete recovery [1]. The ENaC is a multimeric ion channel mostly comprised of three subunit proteins (α, β and γ) and sometimes a δ as well. Attempts have been made to inhibit individual ENaC subunit proteins using antisense technology. Targeting α-subunit of ENaC was selected to knockdown ENaC gene expression due to its critical role in functioning of ENaC [2, 3]. The selection was based on evidence that in single-channel recordings, only the antisense oligonucleotide targeting the α-subunit resulted in a significant decrease in the density of nonselective cation channels. Inhibition of the β- and ϒ-subunit proteins alone or together did not cause any changes in the observed channel density [2]. The reports concluded that α-subunit is critical for the functioning of the channel in respiratory epithelia and thereby knockdown of ENaCα causes a significant decrease in ENaC channel activity. Besides this, Hummler et al. have confirmed the critical role of α-subunit by exhibiting that genetic knock out of α-subunit of ENaC leads to defective lung liquid clearance and premature death in perinatal mice [4].

Studies have been conducted to inhibit the expression of target mRNA using both siRNA and antisense oligonucleotides (AONs). siRNA and AONs though used for similar applications, they differ in many aspects. AONs act through several mechanisms depending on their chemical composition and mostly through induction of RNase H in cytoplasm and nucleus. In contrast siRNA act in a sequential manner through ATP dependent steps leading to irreversible cleavage through RNase III type activity [5]. Our preference for siRNA over AONs was due to their greater resistance to nucleases and 3'-exonuclease compared to AONs making them more efficient and providing long lasting effect [6].

The siRNA is available as predesigned siRNA molecules as well as custom synthesized 21 mer or 27 mer duplexes. The knockdown efficiency of siRNA is claimed to be a function of siRNA sequence such as GC content, design and location of nucleotides at specific places and very much on the degree of siRNA target duplex stability. Therefore, all

these parameters were studied in different predesigned sequences and a final choice was made. The complete characterisation of the predesigned siRNA is provided in subsequent section. The purification of siRNA is done by desalting or HPLC. siRNA was procured as a ready to use HPLC grade duplex in the lyophilized form to be reconstituted in suitable quantity of 1X siMAX^{TM} buffer prepared from supplied 5X buffer (30mM HEPES, 100 mM KCl, 1mM $MgCl_2$, pH = 7.3).

3.2. Characterization of siRNA

The efficacy of siRNA is function of proper design and choice of sequence. Although it cannot be quantitatively justified, confirming some basic design parameter will ensure optimal efficacy for the sequence selected. In most of the cases target site accessibility, free energy of structure equilibrium of siRNA-target hybridization lies as the base criteria for siRNA sequence selection.

The general guideline for selecting target sequences and siRNA sequences are:

1. The siRNA should target mRNA sequence 50-100 nucleotide downstream of ATG start codon.
2. Avoid stretches of 4 or more nucleotide repeats.
3. Avoid sequences which show certain degree of homology with other related or unrelated genes. It can be verified by running blast tool and noting similarity of sequences based on E value (expected value) which should be minimum.
4. Generally, highly functional siRNAs (having functionality more than 95%; ≥F95) have a G-C content that ranged between 36% and 52%.
5. The relative thermodynamic stability and ability to form internal hairpins can be estimated from the predicted melting temperatures (T_m) [7, 8]. The formation of internal hairpin structure can be avoided by choosing sequences with high T_m values. Duplexes lacking stable internal repeats are better silencers. Sorting the functional siRNA classes by T_m shows that no F95 duplexes exhibited $T_m > 60°C$ or predicted hairpin structures.

The siRNA against human ENaCα was received from Empire Bio (Copenhagen, Denmark). The quality of received siRNA was accessed by different analytical

measurements such as Matrix Assisted Laser Desorption Ionization- Time of Flight-Mass Spectrometry (MALDI-TOF) and capillary gel electrophoresis on each single strand of siRNA. Sequence confirmation is an important part for the evidence of the siRNA structure. The MALDI is being used in structural analysis of siRNA and their metabolism and degradation behavior [9]. Capillary Gel Electrophoresis (CGE) is the modification of conventional gel electrophoresis into the capillary containing molecular sieve formed of polymeric solution. The technique allows analytes having similar charge-to-mass ratios to be resolved by size.

3.3. Analytical Method Development

It is essential to ensure that siRNA be analytically evaluated for its quantity, purity and integrity. There are different methods for analytical estimation of nucleotides. The quantity and purity of siRNA has conventionally been estimated using spectroscopic methods due to the absorptivity of purines and pyrimidine. While the integrity is indicator of length and structural intactness of the siRNA and it can be evaluated using electrophoretic mobility assays such as gel electrophoresis. Therefore, it is required that systematic analytical methods be developed for analytical profiling of siRNA.

3.3.1 Materials and instruments

Diethylpyrocarbonate was purchased from Sigma Aldrich, Bangalore; Gel staining solution, Boric acid, Tris buffer was purchased from Himedia laboratories, Mumbai Acetic acid, RNase free glass wares, Nuclease free water, double distilled water etc.

3.3.2 UV Spectrophotometric Analysis of siRNA

Quantification:

Absorption spectrometry has long been the method of choice to measure the amount of DNA and RNA. It is simple, rapid and non-destructive method for quantitative estimation of siRNA. The quantitative estimation of siRNA is based on absorbance of nucleotides at 260 and 280 nm. The absorbance of nucleic acids follows Beer-Lambert law, which predicts linear change in absorbance with concentration. The average extinction coefficient, at wavelength of 260 nm, for dsDNA is 0.020 $(\mu g/ml)^{-1}$ cm^{-1}, for ssDNA it is 0.027 $(\mu g/ml)^{-1}$ cm^{-1}, for ssRNA it is 0.025 $(\mu g/ml)^{-1}$ cm^{-1} and for short single-stranded

oligonucleotides it is dependent on the length and base composition. An OD of 1 equals to ~ 50 ug/ml of dsDNA, ~40 µg/ml of ssDNA and RNA, ~30 ug/ml for single stranded oligonucleotides [10-12].

Purity:

A260/280

Purity of the siRNA was checked by taking the ratio of A260/A280 and A260/A230. The nucleotides that comprise DNA and RNA exhibit widely varying 260/280 ratios. The generally accepted ratios are ~1.8 and ~2.0 for DNA and RNA respectively. The values lower that than of accepted values indicate presence of impurities which absorb at 260 nm such as proteins, phenols and other contaminants. Acidic solutions supress the ratio, while basic solutions over-represent the ratio by 0.2-0.3. RNA will typically have a higher 260/280 ratio due to the higher ratio of uracil compared to that of thymine. It should be noted that the values 1.8 and 2.0 are rule of thumb and the actual ratio depends on the nucleotide compositions.

A260/A230

Due to presence of impurities other than those which absorb at 260 nm, the A260/A230 is used as additional measure for purity determination. The A260/A230 values are usually higher than the corresponding A260/A280 for the sample. The lower values indicate presence of impurities which absorb at 230 nm such as phenol, EDTA, carbohydrates etc.

Nanodrop

The absorption spectroscopy is relatively insensitive and with conventional laboratory setup requires a nucleotide concentration of at least 1 ug/ml. In order to fulfil this requirement, the traditional spectrophotometers cannot be used due to larger sample volumes, therefore, NanoDrop was used to estimate quantity and purity of siRNA samples in our experiments [13, 14]. In addition, NanoDrop measures absorbance at 320 nm to detect any light-scattering components in the sample. The software subtracts the reading at 320 nm wavelength from the 260nm, 280nm and 230nm values as background noise. Once siRNA was confirmed for its purity then a correlation curve was constructed for method

verification using this siRNA. siRNA stock solution was prepared in nuclease free water (NFW):

***Preparation of Nuclease Free Water*:**

Diethylpyrocarbonate (DEPC) is commonly used reagent for preparation of NFW, buffer and other solutions. DEPC is an alkylating agent which reacts with -NH, -SH and -OH groups in RNases and other proteins, resulting in enzyme/nuclease inactivation [15]. Therefore, all the reagents and solutions used in RNA work were prepared in DEPC treated/Nuclease free water.

For this purpose, 0.1% of DEPC was mixed with 1 L doubled distilled water and stirred overnight at room temperature on magnetic stirrer. The dispersion was then autoclaved at 121^0C and 15 psi for 15 min to breakdown the residual DEPC and prepare NFW.

Method verification

Method verification was performed by preparing appropriate dilutions of stock solution (100 pmole/ul) with NFW. siRNA dilutions of various concentrations were prepared using NFW. Absorbance of these solutions as well as stock solution were recorded at 260 nm on a NanoDrop UV spectrophotometer. The method was validated for analytical parameters.

3.3.3 Gel Electrophoresis

In order to maintain bio-activity of siRNA it is essential that the siRNA be delivered in its intact form. The siRNA is highly unstable biomolecule and the intact nature of siRNA can be lost due to hydrolysis. In contrast to DNA, the presence of 2'-OH groups promote RNA hydrolysis under acidic and basic conditions. This results in formation of smaller fragments of siRNA from the initial intact molecule [16]. Therefore, it is vital that the prepared formulation, as well as the developmental process be carried out in presence of a method to detect the impact on the integrity of siRNA.

Electrophoresis is generally used to identify, quantify, and purify nucleic acid fragments and assess quality. The gel electrophoresis works on the principle that when charged molecules are placed in electric field, they travel towards oppositely charged electrode according to their charge. As nucleic acids have a considerable negative charge

due to phosphate backbone, they migrate towards the anode in a matrix of "gel" [17]. The gel run by immersing in an electrophoresis buffer containing ions to conduct current and a buffer to maintain the pH. For RNA applications the gel is composed of agarose at concentrations of 0.5 to 2% for DNA and mRNA analysis. The agarose is a seaweed polysaccharide obtained from Gelidium and Gracilaria, and consists of repeated agarobiose (L- and D-galactose) subunits [18]. The gel can be easily prepared by melting the agarose and pouring it into the slab. During gelation, agarose chains interact non-covalently to form a network of pores which determine a gel's molecular sieving properties. The higher the agarose concentration the "stiffer" the gel. The agarose concentration has to be chosen to suit the particular application.

The detection of siRNA bands can be done by different techniques. The nucleic acid molecules can be visualized under UV light (>2500 µW/cm2) after staining with an appropriate dye. Ethidium bromide is the most common dye used for this application. The ethidium bromide has property to intercalate between the base pairs which results stabilization of the phenyl moiety and a 20 fold increase in fluorescence of orange color corresponding to wavelength ~ 605 nm after excitation with UV light (~305 nm) [19, 20]. Generally, 200 ng of RNA amount is required for visualizing with ethidium bromide. However, the sensitivity of the method can be improved by using stronger fluorescing dyes such as SYBR® Gold and SYBR® Green II RNA gel stain [21]. A UV transilluminator is typically used for this purpose. The light transmitted by fluorescing nucleotide can be detected after blocking the UV light using an orange filter.

The length of RNA or number of nucleotide determines its rate of migration in the gel, the longer RNA molecules move slower than shorter fragments. The loss of integrity is accompanied by siRNA fragmentation and loss of molecular weight. This change can be detected through gel electrophoresis and it is compulsory tool for siRNA quality evaluation. This can also give information about quantity of siRNA. The intact total siRNA when run on polyacrylamide gel produce sharp bands. Partially degraded siRNA appears as smear due to low molecular weight and lack sharp bands. While a completely degraded siRNA forms a very much diffused faint band. If there are secondary structures, the siRNA may not migrate according to its true size resulting in formation of multiple bands for a

single RNA corresponding to different structures. Therefore, inclusion of RNA size marker molecules or control helps in determination of size and interpretation of results and also ensures that the gel was run properly.

Protocol:

The required quantity of agarose was dissolved in 100 ml of 1x TBE (Tris-Borate-EDTA) buffer, by heating with intermittent shaking to obtain a clear solution. Melted agarose cooled just above the pourable consistency. Meanwhile, gel tray for casting was prepared and tightly fastened at both the ends with tape to form a fluid-tight seal and comb was placed in the gel casting tray. To the cooled agarose (45-60°C) Ethidium bromide was (0.5 µg/mL) added before pouring. Ethidium bromide loaded gel was casted with suitable height to contain a well volume of 50 ul. Gel was allowed to set at 20°C for 30 min. After solidification, the comb was removed carefully, without damaging the wells, and tapes were taken off the edges to ensure conductive path in chamber. Gel was submerged in electrophoresis chamber (Genet Electrophoresis Powerpack, Banglore, India) with electrophoresis buffer (1x TBE buffer). Initially, minimum detectable quantity of siRNA on gel was determined by starting from the lowest concentrations: 10, 20, 30, 40, 50 pmole. Then the method for relative quantification based on densitometry was developed and verified by analysing increasing standard concentration of siRNA in the detectable range of 40, 60, 80, 100, 120 pmole. The concentrations were determined relative to reference band of 100 pmole. The method was validated for reproducibility by running 5 multiples and evaluating the RSD.

3.4. Result and discussion:

3.4.1 Characterization of siRNA

The Fig. 3.1 and 3.3 shows the MALDI spectrum of sense and antisense strand of siRNA, respectively. The MALDI provided the confirmation of identity of individual single strand. The Fig. 3.2 and 3.4 shows the capillary gel electrophoresis of sense and antisense strand of siRNA, respectively. The gel electrophoresis provided the information on purity through separation of contaminants and siRNA based on charge and mass selectivity. The purity is

evident from the chromatograph free from impurity peaks. The characterization details of the anti-αENaC siRNA are as fallows:

Human sodium channel alpha subunit

Accession no. NM_001038si.1

- ✓ MW [g/mol]: 13315
- ✓ T_m [°C]: 57.9
- ✓ Purification: HPLC
- ✓ GC-Content [%]: 47.6

Sense strand analysis

Strand sequences (5'→3'): ACUCCA ACCUCUGGAUGUC

- ✓ Target mass of the antisense strand: 6566 Da
- ✓ Detected mass of the antisense strand: 6571 Da
- ✓ Measured purity of the single strand: 93%

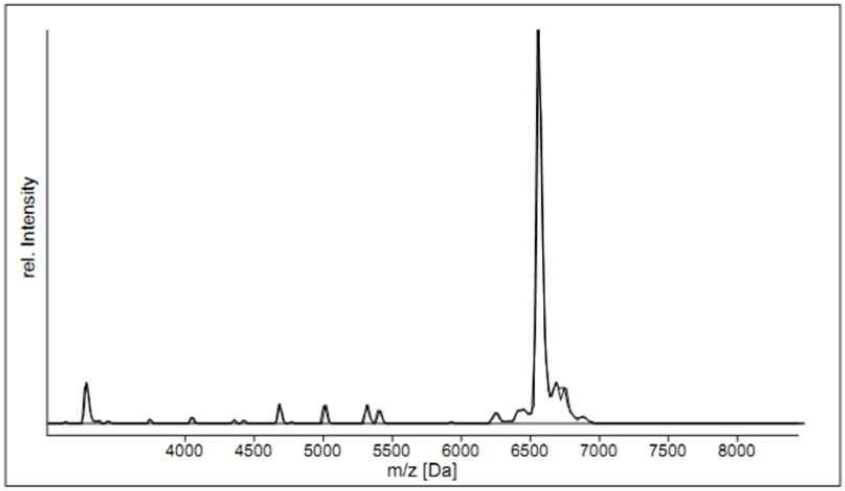

Fig. 3.1: MALDI analysis of sense strand of siRNA showing m/z value

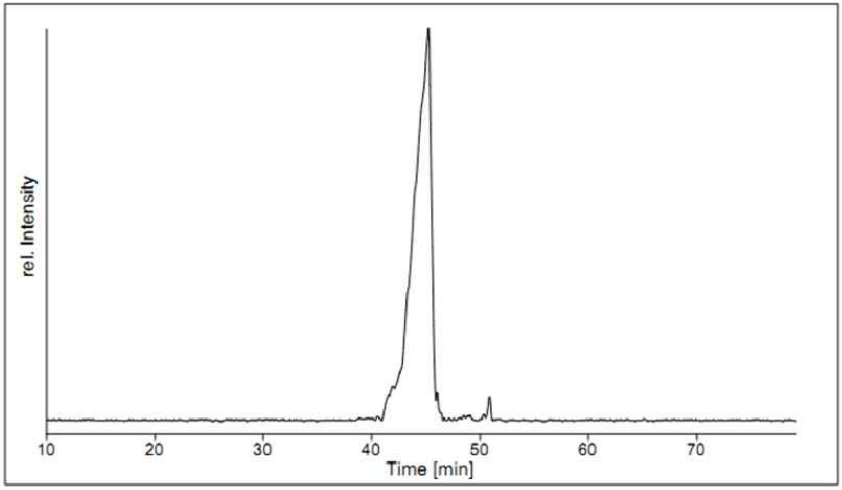

Fig. 3.2: Capillary gel electrophoresis of siRNA showing purity in the form of single peak

Antisense strand analysis

Strand sequences (5'→3'): ACUCCA ACCUCUGGAUGUC

- ✓ Target mass of the antisense strand: 6749 Da
- ✓ Detected mass of the antisense strand: 6750 Da

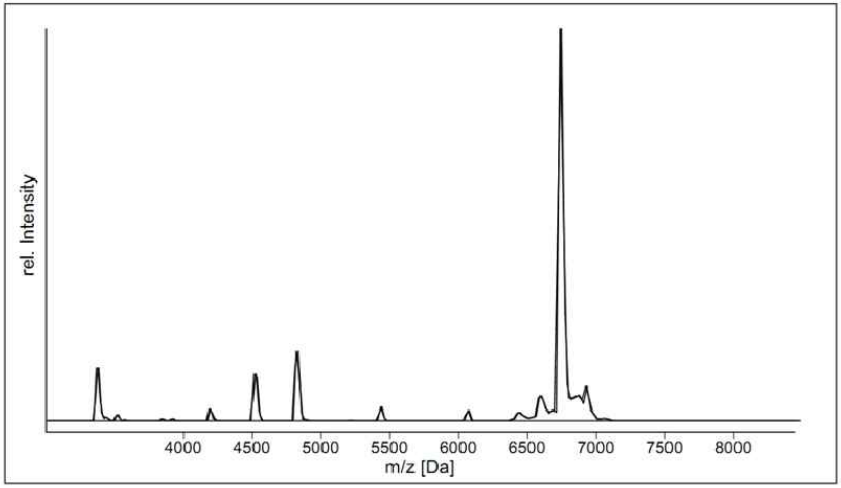

Fig. 3.3: MALDI analysis of antisense strand of siRNA showing m/z value

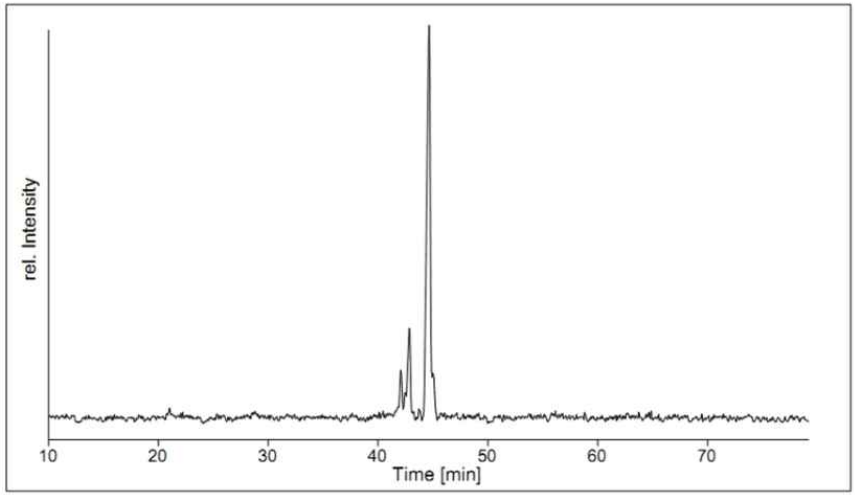

Fig. 3.4: Capillary gel electrophoresis antisense strand of siRNA showing purity

3.4.2 Analytical Methods

Fig. 3.5 shows that the siRNA meets the purity requirements as the A260/A280 and A260/A230 are within the acceptable range. The general sample quantity requirement for experiments were 10 ul, which ensured minimum quantity of siRNA.

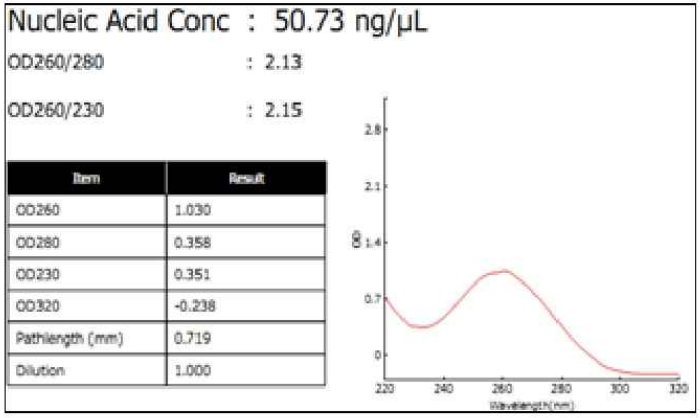

Fig. 3.5: Purity and concentration determination of siRNA using NanoDrop

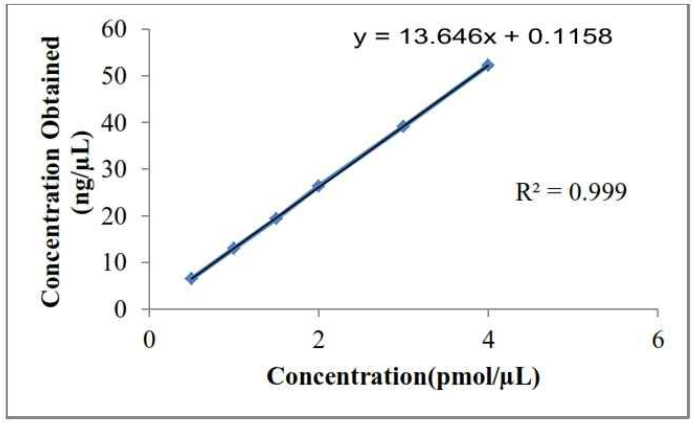

Fig. 3.6: Correlation of known concentration of siRNA Vs NanoDrop results

The correlation between prepared and obtained concentration showed that the method can be used for determination of concentrations from minimum of 0.5 to avoid instrument noise up to 4 pmole/ul to avoid detector saturation as the absorbance was in the range of 0.2 to 1.2 (Table 3.1). This was linearity range for analysis of siRNA by using NanoDrop (Table 3.1 and Fig. 3.6).

Table 3.1: Obtained concentration of siRNA at 260 nm

Concentration of siRNA (pmole/µL)	Obtained Concentration (ng/ µL)	Standard Deviation	% Relative Standard Deviation
0.5	6.87	0.096	1.397
1	13.61	0.138	1.014
1.5	20.514	0.149	0.726
2	27.631	0.182	0.659
3	41.486	0.254	0.612
4	54.34	0.216	0.397

*Values are represented as mean±SD, n=3.

The method verification involved validation for accuracy and precision. Accuracy was defined as closeness of the true and measured readings values of a sample [22]. It was determined from making repeat measurements of three samples concentration. From the

calibration curve three different quality control (QC) levels of drug concentrations were selected [lower quality control sample (LQC) = 0.5 pmole/ul, medium quality control sample (MQC) = 2 pmole/ul, and higher quality control sample (HQC) = 4 pmole/ul]. The samples were prepared from independent stock solution and analyzed in triplicate. Accuracy was quantified by calculating mean percentage recovery and percentage bias. % Bias was calculated as,

$$\% \text{ Bias} = [(\text{predicted conc.} - \text{Nominal conc.})/\text{Nominal conc.}] \times 100.$$

Repeatability was assessed by measuring different QC levels as mentioned in accuracy. To determine intermediate precision inter and intra-day variations was studied. Inter–day variation study was carried out for 3 days. The calculated % RSD of the concentrations was taken as precision [23].

Table 3.2 and 3.3 depict results of accuracy, intraday and inter-day precision of the method respectively. It can be seen that % recovery was found to be in the range of 98.0% to 102.0% which was within the acceptable range. Further, the % RSD values were less than 2% in the precision study and is within the acceptable range as per the requirements of ICH guidelines. Therefore, the method can be used for determination of quantity of siRNA in the sample down to a concentration of 0.5 pmole/ul.

Table 3.2: Results of accuracy measurements

Concentration (pmole/μL)	Obtained Concentration (ng/μL)	Standard Deviation (SD)	%Recovery	% Bias
0.5	6.61	0.088	99.29	-0.71
2	26.68	0.165	100.19	0.19
4	54.11	0.218	101.60	1.60

Values are represented as mean±SD, n=3

Table 3.3: Inter-day precision of the method

	Observed concentration		%Relative Standard Deviation	
Concentration (pmole/µL)	Intraday precision (±SD)	Interday Precision (±SD)	Intraday precision	Interday Precision
0.5	7.08 ± 0.091	7.19 ± 0.075	1.285	1.043
2	27.33 ± 0.125	27.09 ± 0.114	0.457	0.421
4	54.55 ± 0.305	54.69 ± 0.265	0.559	0.485

Values are represented as mean±SD, n=3

3.4.3 Gel electrophoresis

The gel electrophoresis was proposed to study the integrity of siRNA and the complexation capacity of vectors. As the vectors being studied had pH dependent ionization, the electrophoresis buffer pH was decided to be 7.4 as it is physiologically relevant condition. The target was to get sharp bands for pure siRNA. In the first step the agarose concentration was optimized and it was observed that the lower agarose concentrations result in band distortion. When the agarose concentration was increased to 4%, we obtained condensed, crisp band for siRNA. Therefore, the agarose concentration of 4% was finalized for all the experiments.

The pH adjustment of TBE buffer was also important step. The pH of unadjusted 1X TBE buffer is around 8.0. Therefore, it has to be acidified with suitable acid to decrease the pH to 7.4. The pH adjustment with Hydrochloric acid was the direct option. However, it was observed that pH adjusting agent such as HCl, containing ions of higher conductivity, and result in significant increase in conductivity. This increase in conductivity drags the siRNA in its elution path and results in band distortion as shown in Fig. 3.7. Therefore, pH adjusting agent with lower conductivity was proposed for pH adjustment such as Acetic acid. The gel run in electrophoresis buffer containing acetic acid as pH adjusting agent did not result in band distortion which was attributed to the lower conductivity of acetate anions (0.0409 S L mol^{-1} cm^{-1}) compared to chloride anion (0.07635 S L mol^{-1} cm^{-1}). The change in conductivity was apparent from the readings of Ampere on the current supplier at constant voltage of 50 mV.

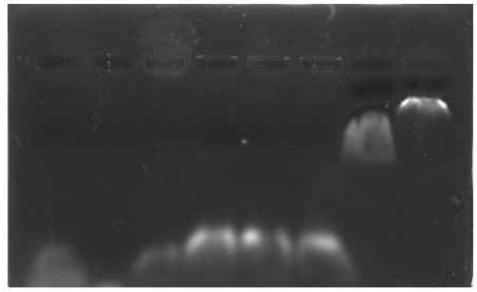

Fig. 3.7: Typical band distortion observed during Gel Electrophoresis

As the method was proposed for evaluating chitosan siRNA complexes to be prepared in acetate or sodium acetate, the sample loading in such vehicles used in formulation may affect the gel electrophoresis. Therefore, siRNA was dispersed in different solvents (0.1% Acetic acid, 0.05% HCl, 0.05% Sodium acetate buffer) and run on the electrophoresis. It was observed that band properties are affected when siRNA is dispersed in vehicle containing ions of high conductivity such as HCl and acidity. At the same time the ionic strength of the vehicle influences the band properties. Fig. 3.8 shows the effect of different vehicles on band quality. It was observed that, to produce good band quality, vehicles with low ionic strength be used e.g. 0.05% sodium acetate rather than 0.1% sodium acetate.

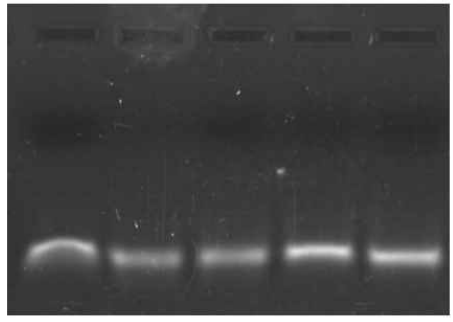

Fig. 3.8: Effect of vehicle of siRNA by gel retardation assay
Lane 1: 1% Acetic acid; Lane 2: 0.1% acetic acid; Lane 3:0.1% Sodium acetate; Lane 4: 0.05% Sodium acetate; Lane 5:NFW.

Stock solution stability: For working with siRNA a primary stock of 500 pmole/ul was prepared from the lyophilized sample. A working solution of 100 pmole/ul was

prepared from the primary stock for routine experiments. As siRNA is highly unstable, the stability of residual siRNA working solution after repeat sampling was assessed. The RNase contamination during sampling can degrade remaining stock, which may result in lower siRNA in subsequent sampling. Therefore, stability of sampled secondary stock against fresh sample from primary stock was checked. Fig 3.9 shows that the siRNA was intact after repeat sampling and remains stable. The table 3.4 shows the summary of the optimized parameters for gel electrophoresis of siRNA.

Fig. 3.9: siRNA stock solution stability to repeat sampling

Table 3.4: Optimized parameters for gel electrophoresis

Parameter	**Optimized condition**	**Comments**
Detectable siRNA	30 pmole	Lower are unsuitable for quantification
Tank buffer	TBE, 7.4 pH	pH adjusted with Acetic acid for low current
Voltage, Current	50 V, 35 mA	Higher voltage caused dragging effect
Vehicles for siRNA	0.05% sodium acetate	Higher conc. resulted in band distortion
Agarose Gel	4% w/w	Lower conc. resulted in drag effect
Run time	40 min	Required for 3/4th run

Quantification:

After optimizing the experimental parameters to get good quality bands, a densitometry method was developed for quantification of siRNA on Gel. First of all, the lowest detectable quantity of siRNA with the optimized Gel Electrophoresis conditions was determined. For this purpose, siRNA at increasing concentration of 10, 20, 30, 40, 50 pmol in NFW and run of electrophoresis. Fig. 3.10 shows that siRNA concentration of >30 pmole is detectable with good visual assessment.

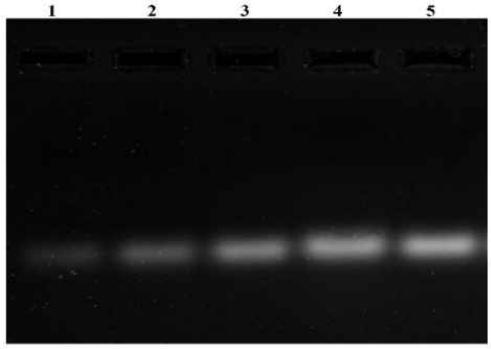

Fig. 3.10: Estimation of minimum quantity of siRNA by gel retardation assay
Lane 1: 10 pmol; Lane 2:20 pmol; Lane 3: 30 pmol; Lane 4:40 pmol. Lane 5:50 pmol.

To enable quantification by densitometry, the relative quantification method was developed and partially validated for analytical parameters. The siRNA concentration of 40, 60, 80, 100 and 120 pmole was run on gel with gel loading buffer and run on 4% agarose gel at 50V.. siRNA was detected with ethidium bromide and visualized on UV transilluminator GelDoc™ XR^+ Imaging System (BioRad, USA). The band density at 100 pmole was taken as reference i.e. 1 and remaining bands were quantified relative to it. The Image lab (version 5.2.1) software was used for densitometry analysis.

Fig. 3.11 shows the band density for different concentrations of siRNA relative to 100 pmole internal standard. The table 3.5 shows correlation between concentration and obtained band density. The RSD values for all the densitometry analysis were < 3.0%. Further, the correlation curve between concentration and relative band density was prepared (Fig. 3.12). It was observed that there was a perfect correlation with R^2 value of

0.996. Therefore, the method is accurate for estimations with use of internal standard in analysis.

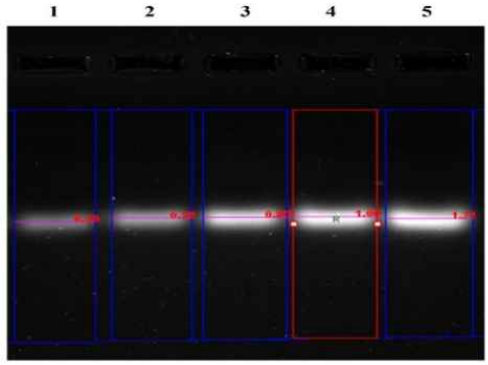

Fig. 3.11: Gel Electrophoresis Band Densities at different siRNA Concentrations
Lane 1: 40 pmol; Lane 2: 60 pmol; Lane 3:80 pmol; Lane 4: 100 pmol; Lane 5:120 pmol.

Table 3.5: Relative band densities at different siRNA concentrations

Concentration of siRNA (pmole)	Relative Band Density	%Relative Standard Deviation
40	0.39±0.011	2.82
60	0.58±0.017	2.93
80	0.80±0.009	1.12
100	1.00±0.018	1.80
120	1.21±0.021	1.73

*Values are represented as mean±SD, n=5.

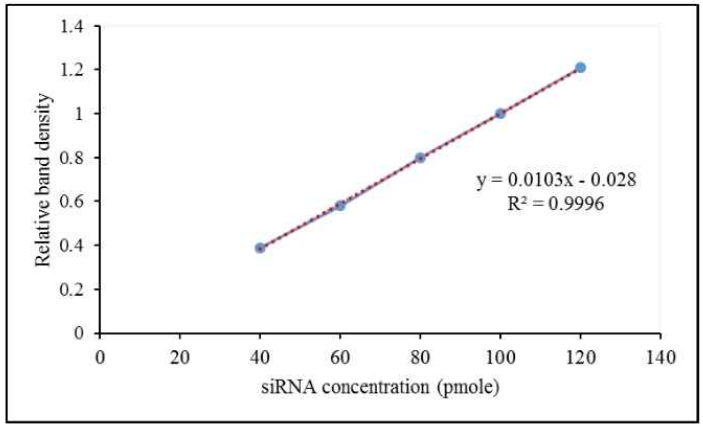

Fig. 3.12: The correlation curve for prepared siRNA concentration and relative band density

Validation

The method was partially validated for analytical parameter of accuracy and precision. The analytical reproducibility was assessed by running the test concentration of 100 pmole in five replicates and using the relative densitometry to determine the concentration of each run (Fig. 3.13).

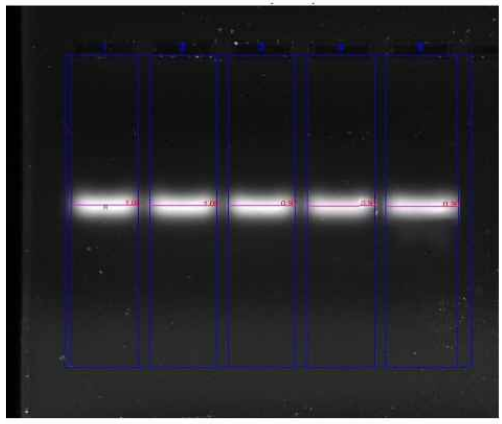

Fig. 3.13: Accuracy and Precision of Gel Electrophoresis Method for siRNA Quantification

The method was also analysed for accuracy and precision. The accuracy was analysed in terms of % recovery and % bias. The % recovery for the method was 98.20

±0.014 and % bias was -1.8%, which was within the acceptable limits. The precision was analysed by % RSD. The % RSD was 1.50 for 5 multiples which was also within the acceptable limits. Therefore, it can be concluded that this method can be used for reliable quantification of siRNA concentrations.

Therefore, the procured human ENaCα siRNA was found to meet the purity requirements as tested by absorption ratios and capillary gel electrophoresis and MALDI data. The agarose gel electrophoresis method was very convenient and highly useful in evaluating the quality and quantity of siRNA. The method was developed to obtain condensed bands to ensure reproducibility and accuracy of results. The method was accurate and precise within the analytical considerations.

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