Pharm Res (2015) 32:806–818
DOI 10.1007/s11095-014-1351-1

RESEARCH PAPER

cRGD Grafted siRNA Nano-constructs for Chemosensitization of Gemcitabine Hydrochloride in Lung Cancer Treatment

Nirav Khatri • Mohan Rathi • Dipesh Baradia • Ambikanandan Misra

Received: 5 February 2013 / Accepted: 27 February 2014 / Published online: 3 May 2014

ABSTRACT

Purpose The aim of present investigation was to effectively deliver ribonucleotide reductase subunit 1 (RRM1) targeted siRNA and assess chemo-sensitization of lung cancer cells against Gemcitabine hydrochloride. It was hypothesised that effective and selective delivery of RRM1 siRNA will help in the treatment of lung cancer chemotherapy using Gemcitabine hydrochloride by reducing drug dose and thereby, reduces dose related toxicity of Gemcitabine hydrochloride.

Methods In this investigation, cRGD grafted siRNA nano-constructs were developed for efficient and targeted intracellular delivery of siRNA. Developed formulations were characterized for gel retardation assay, particle size, zeta potential, cryo transmission electron microscopy, serum stability, in vitro cytotoxicity, qualitative and quantitative cell uptake, gene expression, and chemo-sensitization.

Results Complete complexation of siRNA with cRGD grafted nano-constructs was found at N/P ratio of 2.0. Naked siRNA was found to degrade within 6 h in presence of 50% serum while nano-constructs protected the complexed siRNA even after 24 h. RRM1 level significantly reduced when siRNA was delivered in nano-construct form as compared to naked siRNA. Pre-exposure of RRM1 siRNA decreased the IC_{50} value of Gemcitabine hydrochloride 5 folds in A-549 cells compared to Gemcitabine hydrochloride alone.

Conclusion These results suggest the application of present siRNA delivery strategy to potentiate the chemotherapeutic effect by means of chemosensitization which may be utilized for effective and thorough remission of lung cancer.

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KEY WORDS Chemo-sensitisation · cRGD · Gemcitabine · nano-construct · RRM1 siRNA

ABREVIATIONS

DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DOPE	Dioleoyl-phosphatidylethanolamine
DOTAP	Dioleoyl-trimethylammoniumpropane
DPPC	Dipalmitoyl phosphatidylcholine
DSPE-mPEG$_{2000}$	1,2-Distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (Na+ salt)
DSPG	Disteroyl phosphatidylglycerol
FBS	Fetal bovine serum
HSPC	Hydrogenated soya phosphatidylcholine
L2K	Lipofectamine
MTT	3-(4, 5-dimethylthiazole-2-yl)-2, 5- di-phenyl tetrazolium bromide
RRM 1	Ribonucleotide reductase Subunit

INTRODUCTION

Lung cancer is well characterized by uninhibited cell growth in lung tissues leading to metastases, invasion to adjacent tissue and infiltration beyond the lungs. The two most common histological types of lung carcinoma include Non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). NSCLC generally leads to high mortality and hence proves to be very hostile. Although surgery is a preferred method of cancer removal, it cannot remove the tissue completely and is required to be supplemented by multi-drug chemotherapy and/or radiation as preferred treatment of choice. The chief chemotherapeutic agents used in the treatment of NSCLC are platinum analogs, taxanes, gemcitabine, and vinorelbine etc. However, response to

consequent systemic treatment is approximately 10% for single agents after the failure of initial therapy (1–3). Thus, resistance to systemic therapy does majorly rely on molecular characteristics of individual tumors rather than all-or-none phenomenon.

Ribonucleotide Reductase Subunit 1 (RRM1) gene encodes the regulatory subunit of ribonucleotide reductase, an essential enzyme that catalyses the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides (4, 5). It is the molecular target of Gemcitabine (2, 2-difluorodeoxycytidine), an antimetabolite with activity in several malignancies including NSCLC (6, 7). Previously researchers had suggested that patients with low level of tumoral RRM1 expression had improved survival when treated with Gemcitabine-based chemotherapy as compared to high level of tumoral RRM1 expression (8). In addition, continuous exposure of lung cancer cell lines to increasing amounts of Gemcitabine resulted in increased RRM1 expression. There are proteins residing inside the cells responsible for the activation and metabolism, and thought to be a sole responsible for drug's action. Gemcitabine is activated in cells by nucleoside kinases to Gemcitabine diphosphate (dFdCDP) and Gemcitabine triphosphate (dFdCTP). The cytotoxic effect of Gemcitabine has been attributed to the combination of two actions that lead to inhibition of DNA synthesis and subsequent apoptosis. First, dFdCDP inhibits ribonucleotide reductase which is required for augmenting the reactions that generate the deoxynucleotides for DNA synthesis and repair. Then, dFdCTP competes with dCTP for incorporation into DNA during replication, which results in a termination of chain elongation (9).

RNA interference (RNAi) is the process of mRNA degradation that is induced by double stranded RNA in a sequence-specific manner. RNA interference (RNAi) is a conserved cellular mechanism by which a small double stranded RNA (dsRNA) directs the degradation of complementary mRNA and therefore inhibits the expression of a specific gene (10). Since its discovery, RNAi has become a powerful tool to study gene functions in biological processes (11–13). The ability to induce RNAi in mammalian cells using synthetic small interfering RNA (siRNA) has stimulated great interest in therapeutic applications of RNAi (14–16). In numerous studies, siRNAs have shown promise for treating a variety of diseases, including influenza and HIV infection, cancer and genetic defects (17–19). The double stranded RNA-based molecule, siRNA, has a high potential as therapeutic agent but efficient delivery into target cells is a key challenge in RNAi-based therapy. siRNA is generally having 21 nucleotides and highly charged surface with limited diffusion across the cell membrane. Further, siRNA is prone to degradation by nucleases in the circulation and interstitial space (20). The genetic consequences of cancer strongly support the rationale behind the use of siRNA-mediated gene therapy in the cancer treatment. Numbers of siRNAs have been designed and investigated to target specific malfunctionally regulated oncogenes, or viral proteins involved in carcinogenesis. Furthermore, researchers have envisaged that therapeutic siRNAs can be utilized for silencing target molecules against tumor–host interactions and tumor resistance to chemotherapy and/or radiotherapy (21).

During the past few years, cRGD peptides have become very popular agent for targeting of therapeutics and imaging agents to cancer tissue over expressing integrin. Various chemical modifications have been applied to attach cRGD peptides and its modified forms to liposomes, polymers, peptides and radiotracers. cRGD grafted liposomes have been investigated as an impending carrier for tumor targeting of chemotherapeutics. The present research work was aimed to develop stable siRNA nano-constructs using suitable lipid carrier and chemosensitization of the anticancer agent Gemcitabine HCl by pre exposure of siRNA encapsulated nano-constructs. cRGD conjugated siRNA nano-constructs were used to target non-small cell lung cancer.

MATERIAL AND METHODS

Chemicals

Dioleoyl-trimethylammoniumpropane (DOTAP), Dioleoyl-phosphatidylethanolamine (DOPE), Hydrogenated soya phosphatidylcholine (HSPC), Dipalmitoyl phosphatidylcholine (DPPC), Disteroyl phosphatidylglycerol (DSPG) and 1,2-Distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-mPEG$_{2000}$) were purchased from Lipoid GmbH (Germany). cRGD-PEG$_{2000}$-DSPE was purchased from Peptide International (USA). Cholesterol and ethidium bromide were purchased from Sigma-Aldrich (USA). Agarose, Tris base, boric acid, ethylenediamine tetra acetic acid, Diethyl pyrocarbonate (DEPC), fetal bovine serum (FBS), penicillin, streptomycin were procured from Himedia lab. Pvt. Ltd. (Mumbai, India). Chloroform and methanol were purchased from SD Fine chemicals Ltd. (Mumbai, India). All other solvents used were of analytical or HPLC grade.

siRNA targeted to human RRM1 mRNA was synthesized and purified with HPLC by Eurofins MWG Operons Ltd (Germany). Carboxyfluorescein (FAM) labelled negative control siRNA (FAM-NC-siRNA) and FAM labelled negative control (scramble) siRNA were obtained as a gift sample from GenePharma (Shanghai, China).

Cell Line

The human lung carcinoma cell line (A549) was procured from NCCS Pune. A549 cells were cultured in Dulbecco's

Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 incubator (IGO150, Jouan, Germany).

Preparation of Nano-construct

siRNA nano-constructs were prepared using lipid based non-viral delivery vectors. siRNA encapsulated nano-constructs were prepared by incubating siRNA with preformed liposomes. Briefly, liposomes were prepared by film hydration method. Combinations of lipids viz. DOTAP, DOPE, HSPC, Cholesterol, $mPEG_{2000}$-DSPE and cRGD-PEG_{2000}-DSPE were dissolved in 3 ml of chloroform in a 50 ml round bottom flask at different molar ratios and organic solvent was evaporated under vacuum (600 mmHg) and temperature (45°C) using rotary evaporator (IKA RV-10, USA). The thin lipid film was hydrated by 5 ml of DEPC treated nuclease free water at 60°C. The size of liposomes was reduced using successive extrusion through 1, 0.4, 0.2 and 0.1 µm polycarbonate membranes (Whatman, USA) using high-pressure extruder (Avestin, USA). siRNA was incubated with these preformed liposomes at different N/P ratio, ranging from 0 to 2.0, for half an hour at 37°C under constant stirring. Various combinations of lipids were utilized to optimize the formulation with regard to encapsulation of siRNA.

Gel Retardation Assay

Following incubation period, siRNA nano-constructs were subjected to gel electrophoresis to assess encapsulation of siRNA within preformed liposomes. siRNA nano-constructs were mixed with 2 µl of 6X DNA gel loading buffer (Fermentas Life Sciences, USA) and loaded onto a 2% agarose gel containing 0.5 µg/ml ethidium bromide, and separated by electrophoresis for 20 min at 100 V in TBE buffer (10.8 g/l Tris base, 5.5 g/l boric acid and 0.58 g/l EDTA). Afterwards, siRNA was visualized by UV transillumination and gel photography using a Gel Doc System (Bio-Rad Lab., USA).

Particle Size and Zeta Potential

The average particle size and zeta potential of siRNA nano-constructs were determined by differential light scattering with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Prior to the measurement siRNA nano-constructs were diluted with nuclease free water and measurements were carried out at 25°C. Zeta potential was calculated by Smoluchowski's equation from the electrophoretic mobility. Each sample was measured three times and the mean values were calculated.

Cryo-Transmission Electron Microscopy (Cryo-TEM)

Morphology and lamellarity of the lipoplex were studied using Cryo-TEM (TECNAI G2 Spirit BioT WIN, FEI-Netherlands) operating at 200 kV with resolution of 0.27 nm and magnifications of the order of 750,000X. Hydrophobic carbon grid was converted to hydrophilic nature by using Glow Discharge to perform cryo TEM (Emitech K100X, Quoram Technologies, UK). Formulation was evenly dispersed on prepared grid and the grid was cryo-freezed in liquid ethane at -180°C. Cryo-freezed grid was transferred to cryo-holder maintained at -175°C using Liquid Nitrogen storage box. The cryo-holder was then inserted in the microscope for imaging the sample. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form, lamellarity and vesicle size of the prepared formulation.

Serum Stability

The siRNA nano-constructs were studied for the integrity of complexed siRNA in presence of serum for possible *in vivo* degradation because of extracellular and intracellular RNAse. Nano-constructs containing 2.6 µg of siRNA were incubated with 50 µl non-heat inactivated FBS at 37°C for various time periods to give a 50% serum concentration. Nuclease free water was then added to give a final volume of 100 µl. The nano-constructs were then broken with chloroform (50 µl) and the siRNA was back extracted in water by vortexing followed by centrifugation. The siRNA was purified by phenol-chloroform extraction and was precipitated by 70% ethanol. After re-dissolving the siRNA in minimum quantity of DEPC treated water, the siRNA was run on 2% agarose gel to check the integrity of siRNA and protection offered by nano-constructs against serum.

In-vitro Cytotoxicity Assay (MTT Assay)

The cytotoxicity of siRNA carriers was determined using 3-(4, 5-dimethylthiazole-2-yl)-2,5- di-phenyl tetrazolium bromide (MTT; Himedia, India) assay. Cells to be assayed were seeded onto 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were treated separately with nano-constructs at the N/P ratio ranging from 2.5 to 12.5 and commercially available non-viral lipid transfecting carrier Lipofectamine-2000 (L2K; Invitrogen, USA) in DMEM containing 10% FBS and antibiotic. After 6 h, transfection media was replaced by fresh DMEM containing 10% of FBS and antibiotics. The cells were incubated for 48 h, and then 20 µl of 5 mg/ml MTT solution was added to each well. After incubating for 4 h, the culture medium was removed and 200 µl of a DMSO (Sigma, USA) was added. The reduction of viable cells was measured by colorimetry at 570 nm wavelength using an

enzyme-linked immunosorbent assay plate reader (Bio-Rad, USA). Cell viability of each group was expressed as a relative percentage to that of control cells.

In-vitro Cell Uptake Studies

For cellular uptake studies, FAM labelled negative control siRNA (FAM-NC-siRNA) was used. Confocal microscopy was utilized for qualitative cell uptake while quantitative intracellular accumulation was determined using flow cytometry.

Flow Cytometry

A549 cells were seeded at a density of 5×10^5 cells per well in 24 well plates. After 24 h proliferation, nano-constructs containing FAM-NC-siRNA at a final concentration of 100 nM were exposed to cells and incubated for additional 6 h at 37°C in humidified air with 5% CO_2. After incubation, the cells were harvested and washed three times with cold phosphate buffer solution (PBS) and then analysed for mean fluorescence activity using fluorescence activated cell sorter (FACS-BD-AriaIII, BD, USA). Naked FAM-NC-siRNA and L2K complexed siRNA were used as negative and positive control respectively.

Confocal Microscopy

Cellular internalization of siRNA in A549 cells was monitored by confocal microscopy. Cells were seeded onto 6-well plates at a density of 10^4 cells/well with a glass cover slip at the bottoms. After 24 h, cells were transfected with FAM-NC-siRNA containing nano-constructs at a final concentration of 100 nM. After 6 h incubation, cells were washed with cold PBS immediately and fixed using ice cooled 4% paraformaldehyde solution for 10 min., followed by cell nuclei staining with DAPI for 10 min. Cover slips were mounted on slides after washing with PBS three times and proceeded for confocal microscopy using confocal laser scanning microscope (LSM 710, Carl-Zeiss Inc., USA).

Live imaging was performed using confocal microscopy. 5×10^4 cells were seeded onto confocal microscopic petridish with glass cover slip (Nunc, India). After 24 h cells were transfected with FAM-NC-siRNA at 100 nM concentration. Soon after transfection imaging was started. Furthermore, the lateral Z- stack images were constructed during imaging from the middle zone of the cells.

Transfection Studies

siRNA mediated transfection was studied by quantifying mRNA knock-down using real time PCR. A day before transfection, A-549 cells were seeded onto 24 well plates at a density of 5×10^4 cells/well. After approximately 80% confluency, cRGD grafted RRM1 siRNA nano-constructs were added to each well. Three different concentrations of siRNA were used i.e., 50 nM, 500 pM and 50 pM. Cells were also transfected with commercially available L2K according to the manufacturer's instruction. After 48 h, total RNA was isolated using the TRIzol reagent (Invitrogen, USA) and reverse transcribed into cDNA using RNA to cDNA conversion kit (Invitrogen, USA). The mRNA level was quantified using Step One real time PCR (Applied Biosciences, USA). Each reaction contained SYBR Green Master mix (Applied Biosciences, USA), forward and reverse primer, and 2 ng of cDNA in a total volume of 20 µl. The primers for RRM1 siRNA were 5′-TGAGCAGCGCCTGGAACCTAA-3′ for forward and 5′-GCATCGCAGCTAGTGGCTGA-3′ for reverse (PCR product 117 bp). The mRNA expression level of RRM1 was normalized against housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Chemosensitization Studies

In vitro cytotoxicity of anticancer drug Gemcitabine HCl at sequential concentrations was assessed with pre-treatment of cRGD grafted siRNA nano-constructs in A549 cells. Gemcitabine HCl solution (Gemcitabine solution) and Gemcitabine HCl liposomes (Gemcitabine liposome) were used as chemotherapeutic agents. Gemcitabine solution was obtained by reconstituting lyophilized injection of Gemcitabine HCl with saline solution. Lyophilized injection was composed of Gemcitabine HCl, sodium acetate and mannitol. Gemcitabine liposome were prepared from DPPC, DSPG, cholesterol, $mPEG_{2000}$-DSPE (5.6: 2: 2: 0.4) with mean particle size of 150 nm.

Aliquots of 10^6 cells were seeded in 60 mm dishes. After 24 h proliferation, the cells were transfected with siRNA nano-construct in antibiotics and serum free medium. The final concentration of siRNA was 50 pM. After 6 h transfection, the culture medium was replaced with fresh DMEM supplemented with 10% FBS and antibiotics. Following next 42 h of incubation, cells were harvested and seeded in 96-well plates at a density of 5×10^3 cells per well. After 24 h proliferation, cells were treated with a series of concentrations of Gemcitabine solution and Gemcitabine liposomes for 48 h, and 20 µl of a 5 mg/ml MTT was added to detect IC_{50} values.

STATISTICAL ANALYSIS

Statistical analysis of data was performed using an ANOVA and Student's t-test. GraphPad Prism (version 5, USA) was used for all analyses and P value <0.05 was considered significant.

RESULTS

Nano-constructs were prepared by incubating cationic liposomes with siRNA at an optimal condition to form a complex between quaternary nitrogen of DOTAP and phosphate of siRNA molecule (Table I). N:P was calculated only on the basis of cationic charge of DOTAP and anionic charge of siRNA. Nano-constructs formed via mixture of DOTAP and DOPE was very flexible. Cholesterol and subsequently neutral phospholipid, HSPC, were used to form rigid complex and to provide physical stability. The gel retardation pattern of siRNA was affected by the N/P ratios of lipid and siRNA, amount of cholesterol, and ratio between cationic to neutral lipids. Figure 1 show that nano-constructs formed by incubating preformed liposomes made up of DOTAP, DOPE, Cholesterol and HSPC (DDCH) completely inhibited the electrophoretic mobility of siRNA at N/P ratio of 2.0. Below N/P ratio of 1.0, considerable amount of the siRNA migrated as free siRNA on agarose gel towards positive electrode. Incorporation of 3 mol% $mPEG_{2000}$-DSPE and 2 mol% cRGD-PEG_{2000}-DSPE into prepared liposomes showed same result for siRNA encapsulation (Fig. 2). The optimized siRNA nano-constructs delivered siRNA more effectively and with lower cytotoxicity than conventional L2K.

Previous study has suggested that the transfection efficiencies of cationic liposomes/lipoplexes are affected by several parameters, including the size and structure of the siRNA complexed liposomes and their cytotoxicity (22). Without siRNA, the mean size of cRGD grafted nano-constructs was 98.2±2.0 nm with PDI value 0.121±0.04. However, after forming complex with siRNA at the N/P ratio of 2.0, the average size of cRGD grafted siRNA nano-constructs was increased to 132±1.3 nm with PDI value 0.174±0.05 (Fig. 3a). Zeta potential of the same formulation was 35.3±0.3 mV before complexation and 12.17±0.6 mV after complexation to siRNA (Fig. 3b) at the N/P ratio of 2.0. Images revealed that prepared nano-constructs are spherical in shape as shown in Fig. 4. All vesicles are unilamellar in structure and having particle size between 150 and 200 nm.

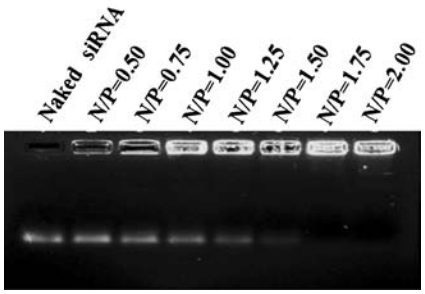


Fig. 1 Gel electrophoresis of DDCH nano-constructs at different N/P ratios.

An essential property of nano-constructs designed for further applications is the ability to protect the encapsulated siRNA from degradation by serum nucleases (mainly RNAse). Therefore, we determined the serum stability of our cRGD grafted siRNA nano-constructs by incubating them in FBS at 37°C. Gel retardation assay showed that degradation was started within half an hour after incubation of siRNA with FBS. At 0.5 h more than 10% of siRNA was degraded, which reached up to 60% within 3 h. After 6 h only 19.3% of siRNA was remained as compared to initially loaded siRNA. cRGD grafted nano-construct encapsulated siRNA was stable even after 24 h and more than 90% of siRNA was seen on agarose gel (Fig. 5a and b). This result indicates that prepared nano-constructs successfully protected the encapsulated siRNA from enzymatic degradation.

In-vitro Cytotoxicity Assay (MTT Assay)

In vitro cell line studies for the cytotoxicity of prepared nano-constructs were thoroughly carried out. It was seen that at N/P of 2.5 DDCH (composed of DOTAP, DOPE, HSPC and Cholesterol) and cRGD grafted siRNA nano-constructs were significantly less toxic than L2K. Even at higher N/P ratio of 12.5 at 100 nM siRNA concentrations these nano-constructs were non-significant in toxicity as compared to L2K. Graphical representation of cell viability against increasing ratio of N/P i.e., charge ratio of cationic lipid to siRNA after treatment with developed nano-constructs was obtained by MTT assay as shown in Fig. 6. Cytotoxicity of blank was

Table I Optimized Formulation of Nano-constructs

Sr No	Formulation	Lipids	Ratio	Lipid (mg/mL)	Total lipid (mg/ml)
1	DD nano-constructs	DOTAP: DOPE	1:1	DOTAP-2.84 DOPE-2.84	5.68
2	DDC nano-constructs	DOTAP: DOPE: Cholesterol	1:1:0.3	DOTAP-2.84 DOPE-2.84 Cholesterol-4.0	9.68
3	DDCH nano-constructs	DOTAP: DOPE: Cholesterol: HSPC	1:1:2.7:3.3	DOTAP-2.84 DOPE-2.84 Cholesterol-4.0 HSPC-10.0	19.68
4	RGD grafted siRNA nano-constructs	DOTAP: DOPE: Cholesterol: HSPC: $mPEG\text{-}DSPE_{2000}$: RGD-$mPEG\text{-}DSPE_{2000}$	1:1:2.7:3.3:0.24:0.16	DOTAP-2.84 DOPE-2.84 Cholesterol-4.0 HSPC-10.0 $mPEG\text{-}DSPE_{2000}$ – 2.50 cRGD-$mPEG\text{-}DSPE_{2000}$—2.14	24.32

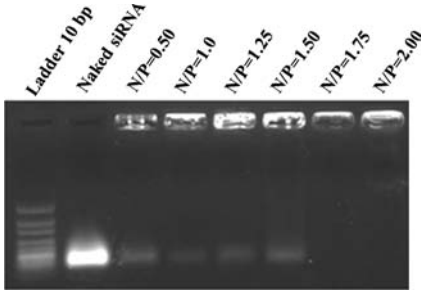


Fig. 2 Gel electrophoresis of cRGD Grafted siRNA nano-constructs at various N/P ratios.

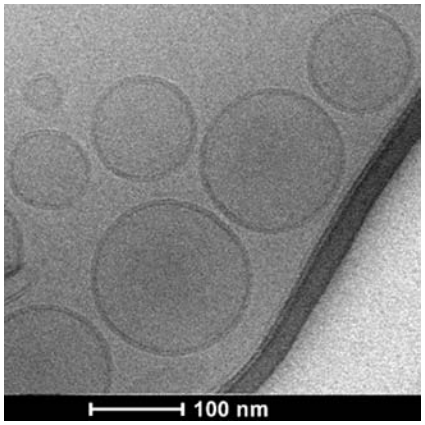


Fig. 4 Cryo-TEM of cRGD grafted nano-constructs.

used as background. DDCH and cRGD grafted siRNA nano-constructs reduced viability to 85.6±2.1% and 89.4±2.2% respectively at N/P ratio of 2.5, while L2K reduced viability to 79.4±1.7% following 48 h of incubation. At 12.5 N/P charge ratio DDCH and cRGD grafted siRNA nano-constructs showed 79.8±1.4% and 77.8±4.2% cell viability respectively. Amongst the nano-constructs studied, DDCH and cRGD grafted siRNA nano-constructs showed the highest cell viability at all charge ratio.

In-vitro cell uptake

cRGD motifs have higher affinity to NSCLC cells comparing to normal epithelial cells because αvβ3 integrin is generally found to be overexpressed in tumor biopsy samples but not in normal tissues. Expression of integrins is often correlated with disease progression in various tumor types. cRGD targeted nanocarriers for gene delivery is a

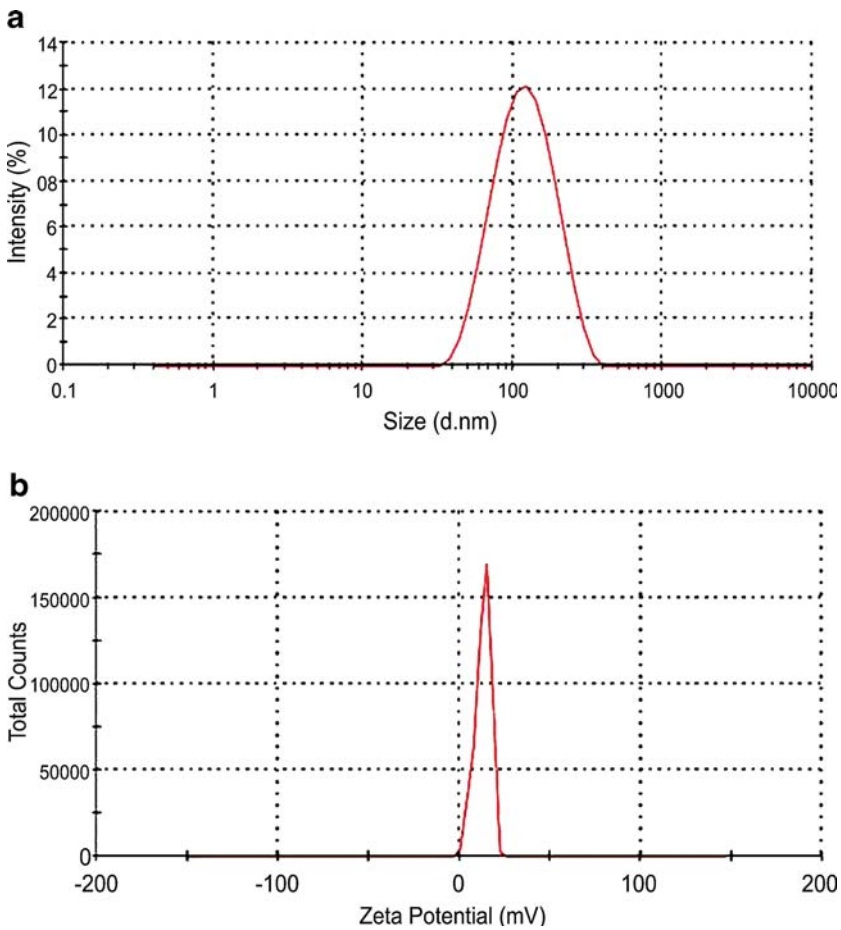


Fig. 3 (**a**) Particle size of cRGD grafted siRNA nano-constructs. (**b**) Zeta potential of cRGD grafted siRNA nano-constructs.

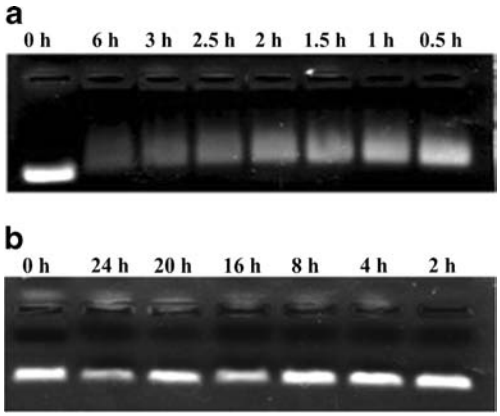


Fig. 5 (**a**) Serum stability of naked siRNA by gel retardation assay. (**b**) Serum stability of siRNA in cRGD grafted nano-constructs by gel retardation assay.

relatively new approach and enhancement of *in vitro* and/or *in vivo* gene transfer has been observed in numerous reported studies. As shown in Fig. 7a and b, the order of fluorescence intensity in cells after treatment with various siRNA formulations was as follows: free siRNA < DD nano-constructs (composed of DOTAP and DOPE) < DDC nano-constructs (composed of DOTAP, DOPE, Cholesterol) < DDCH nano-constructs (DOTAP, DOPE, HSPC and Cholesterol) < cRGD grafted siRNA nano-constructs. It was suggested from this result that nano-constructs could significantly enhance siRNA translocation into cells when compared to that of negatively charged free siRNA. Furthermore, DDCH and cRGD grafted siRNA nano-constructs showed greater mean fluorescence intensity (83.83±1.7 and 88.16±2.9) than L2K complexed siRNA (74.63±2.4) at N/P ratio of 2.0. These results were supported by qualitative analysis where intracellular localization of FAM-NC-siRNA (green) was investigated using laser confocal microscope as shown in Fig. 8. After 6 h incubation, FAM-NC-siRNA was mainly observed in cytoplasm with a relative uniform distribution. Live images with Z-stacking (Fig. 9) shows marginal different pattern of intracellular localization with cRGD grafted siRNA nano-constructs as compared to DDCH nano-constructs and naked siRNA.

Transfection Studies

Using optimized transfection conditions, cells were transfected at final siRNA concentration of 50 nM, 500 pM and 50 pM. cRGD grafted siRNA nano-constructs showed higher mRNA knock down ($p<0.05$) than naked siRNA (Fig. 10). At 50 nM concentration nano-constructs down regulated mRNA level upto 26.15±1.2 while naked siRNA exhibited 83.50±3.5 down regulation. There was no significant difference ($p>0.05$) between nano-construct and L2K (26.35±2.1) at 50 nM concentration. At lower concentrations, 500 pM and 50 pM, inhibition was markedly decreased with both nano-constructs (74.65±4.7 and 88.05±2.8) and L2K (72.15±4.1 and 91.50±2.1). Naked form demonstrated very poor gene silencing at lower concentrations i.e., 99.65±4.7 and 97.6±7.9 respectively for 500 pM and 50 pM. Results suggest that 50 pM is the sub-growth inhibitory concentration which was utilized for further Chemosensitization studies.

Chemosensitization Studies

MTT assay was used to determine IC_{50} values of Gemcitabine HCl in A549 cells pretreated with cRGD grafted siRNA nano-constructs at final RRM1 siRNA concentration of 50 pM. siRNA pre-treated Gemcitabine liposomes and siRNA pre-treated Gemcitabine solution exposed cells showed less IC_{50} values, 207.6±1.8 ng/ml and 227.6±3.8 ng/ml respectively (Fig. 11). These values are significantly less as compared to IC_{50} values of cells treated with Gemcitabine liposomes and Gemcitabine solution only i.e., 722.6±11.7 and 1088.2±15.7 respectively. Results strongly suggest the Chemosensitization effect by pre-exposure of siRNA nano-constructs by 5.2 fold when siRNA-Gemcitabine liposome was used as compared to Gemcitabine solution, where siRNA nano-constructs were pre-exposed at pM concentration (Tables II and III). At sub-inhibitory concentration i.e. 50 pM, it was found that cancer cells were significantly sensitized to the treatment of Gemcitabine. RRM1 is a major cellular determinant of cytotoxic efficacy of Gemcitabine. Pre-exposure of siRNA formulation incurred the cascade of chemosensitization which resulted in

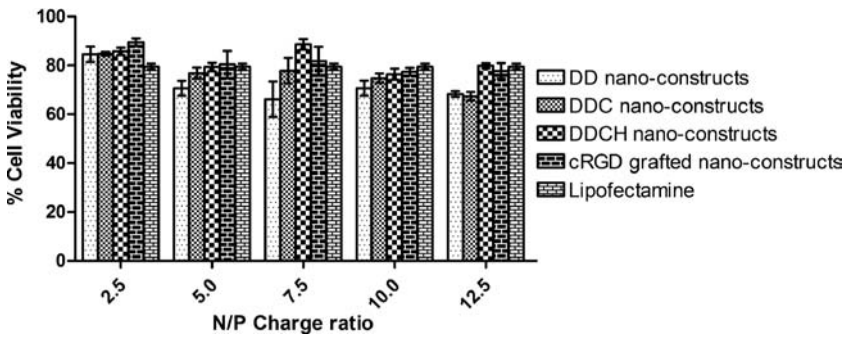
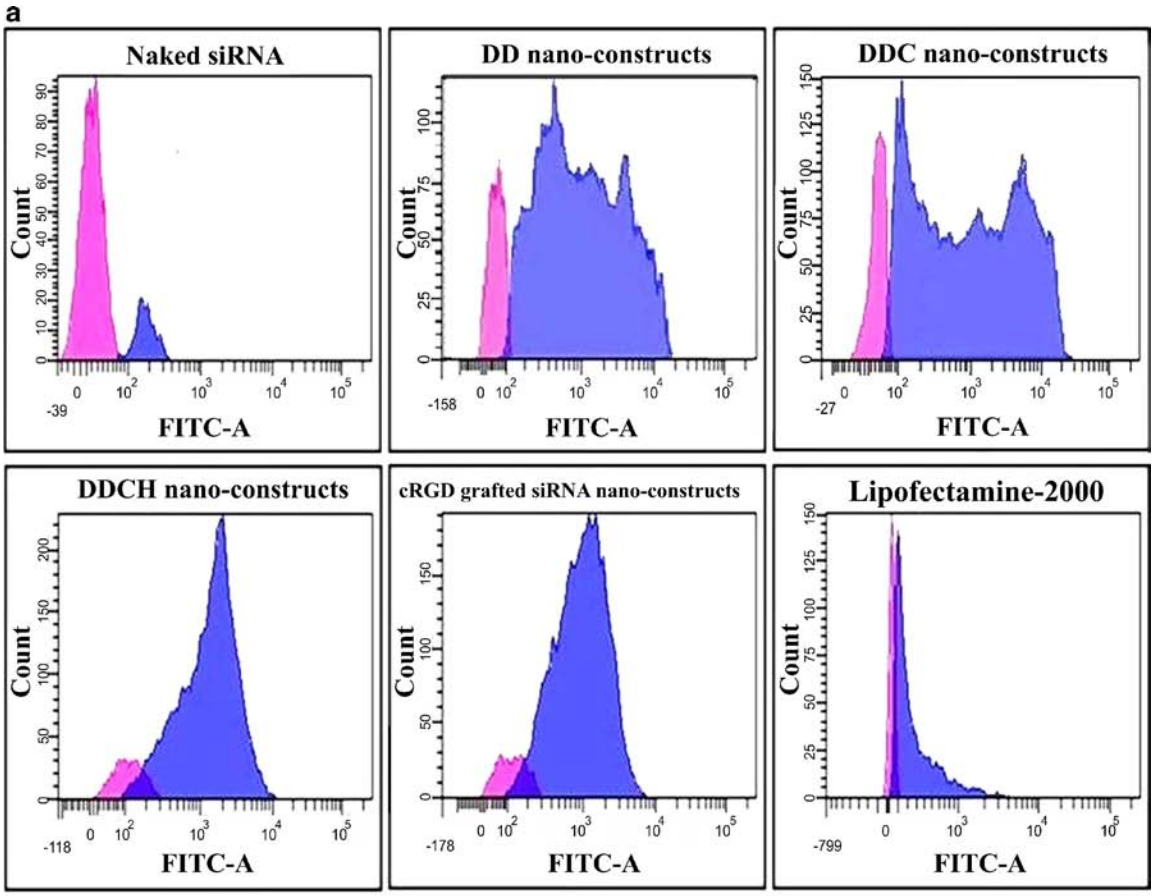
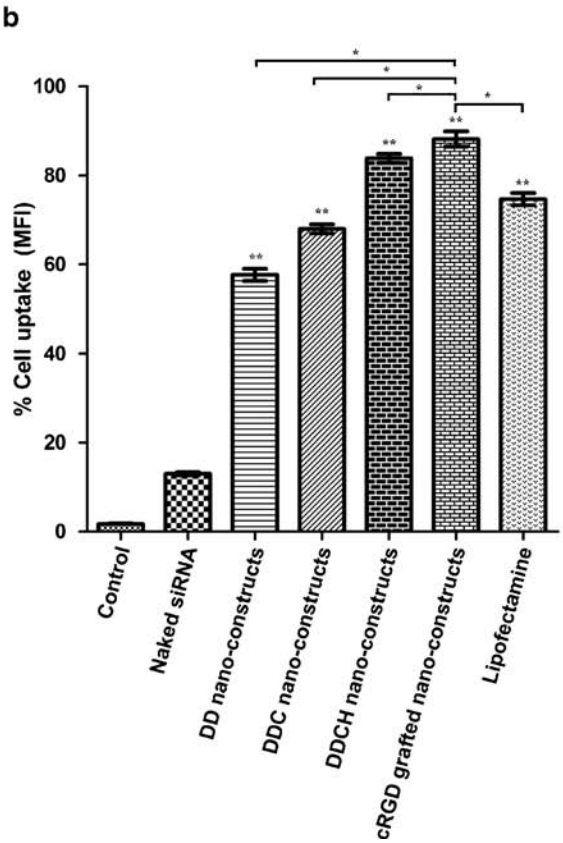


Fig. 6 Cell cytotoxicity studies of developed nano-constructs in A549 Cells.


a
Naked siRNA
DD nano-constructs
DDC nano-constructs
DDCH nano-constructs
cRGD grafted siRNA nano-constructs
Lipofectamine-2000
Count
FITC-A


b
% Cell uptake (MFI)
Control
Naked siRNA
DD nano-constructs
DDC nano-constructs
DDCH nano-constructs
cRGD grafted nano-constructs
Lipofectamine

◀ **Fig. 7** (**a**) Quantification of cell uptake by flow cytometry in A549 cells. (**b**) Mean Fluorescence Intensity (MFI) of cells obtained by flow cytometry, Population: 10,000 cells, FAM-NC-siRNA: 100 nM. * indicates significant difference ($p < 0.05$) when analyzed with paired t-test of two samples for means. ** indicates statistically significant difference ($p < 0.005$) when compared to naked siRNA.

reduced protein level of RRM1 and ultimately turned to improved inhibitory activity after treatment with Gemcitabine.

DISCUSSION

One of the study previously has reported the resistance to Gemcitabine in two NSCLC cell lines (H358 and H460), generated *in vitro* by exposure of increasing concentrations of the drug and found that resistance is principally a function of increased expression of RRM1 (7).

Many cationic lipids have been previously used as delivery vehicle for plasmid DNA and siRNA (23, 24) but there are severe toxicity related issues with use of these lipids. However, incorporation of some neutral lipids with an optimal amount may limit this disadvantage. In present investigation, we developed cationic liposomes using DOTAP as a key lipid, bearing positive charge, for encapsulation of negatively charged siRNA. However, other helper lipids included into formulation maintained the rigidity, fusogenicity and other vital functions of developed nanoconstructs. DOPE is extensively used as a fusogenic lipid in combination with cationic lipids for cellular delivery of siRNA and proteins (25).

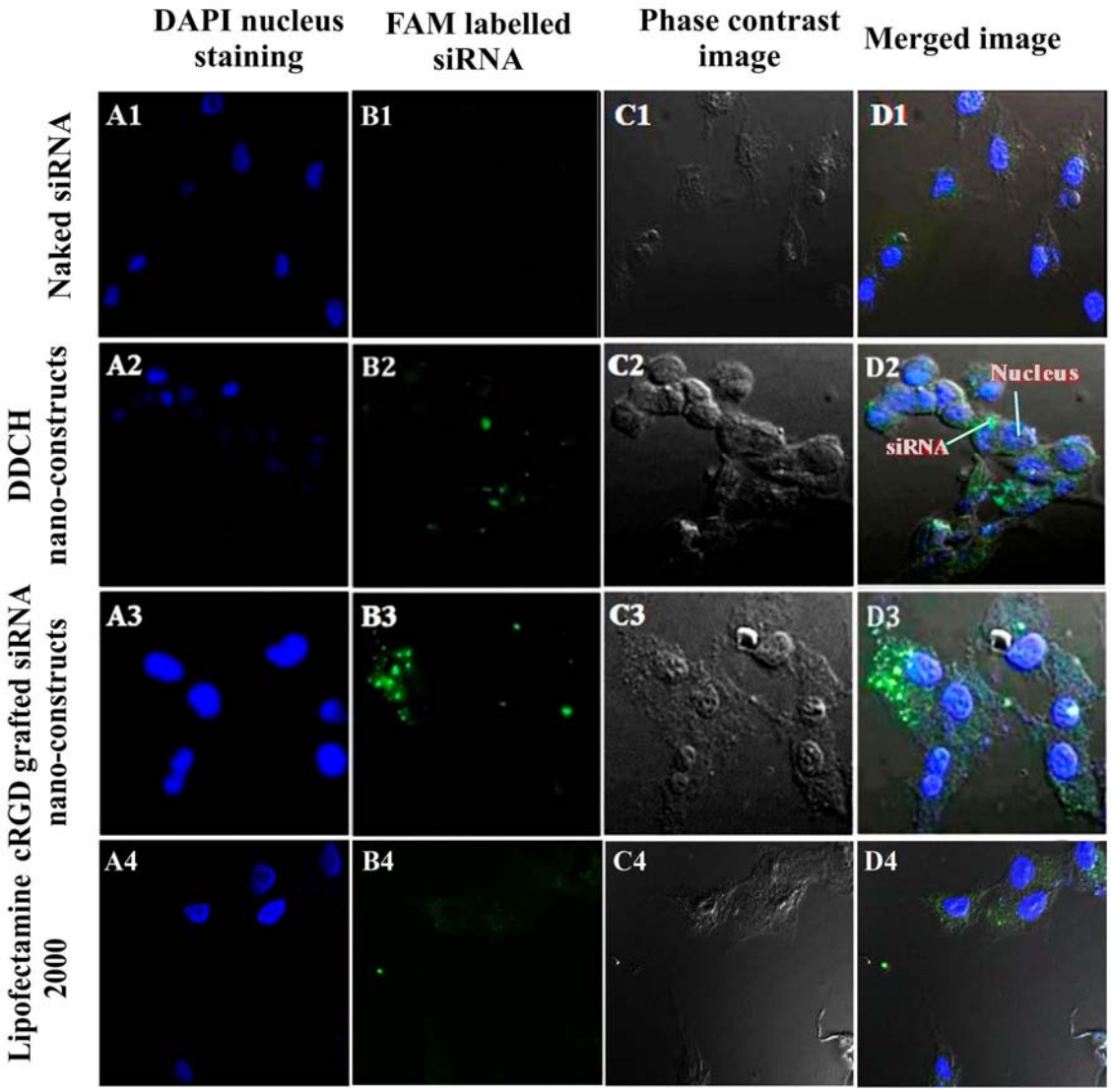


Fig. 8 Intracellular localization of FAM-NC-siRNA in A549 cells by confocal microscopy. A: DAPI Nucleus staining, B: FAM labeled siRNA, C: Phase Contrast, D: Merged Image. A1, B1, C1, D1-Naked siRNA; A2, B2, C2, D2-DDCH nano-constructs; A3, B3, C3, D3-cRGD grafted siRNA nano-constructs; A4, B4, C4, D4-Lipofectamine 2000 (L2K).

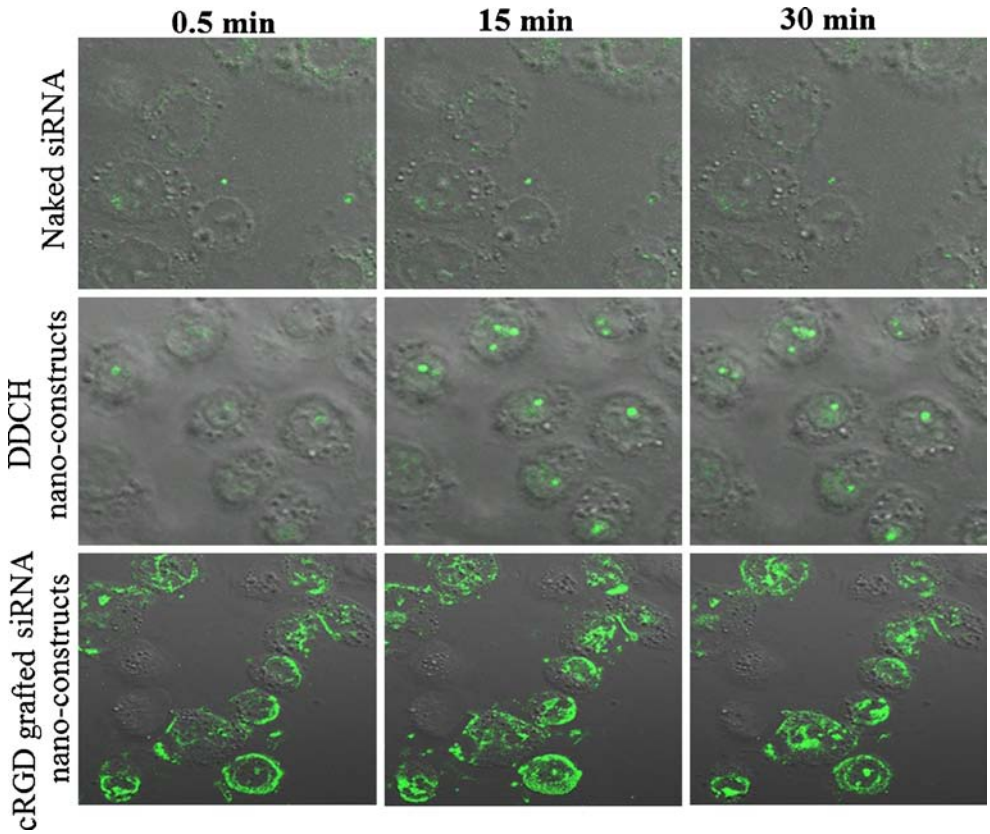


Fig. 9 Live images of intracellular localization by confocal microscopy.

Currently a study reported that incorporation of DOPE with cationic cholesterol derivatives can provide stability to siRNA against serum (26, 27). DOPE in liposomal membranes is thought to preferentially form the inverted hexagonal phase, a phase typical of membrane–membrane fusion events (28). Many researchers have shown that cRGD grafted carriers may be promising tool for tumor targeting, over expressing the integrin receptors on cell surface of tumor. It was demonstrated that addition of cRGD moieties leads in receptor mediated endocytosis in cancerous cells and partly increase the transfection. DDCH formulation does not have any selectivity for NSCLC and it may give off-target effects in normal cells, and that's why cRGD has been used as a target moiety in present work to enhance the specificity of delivery system towards cancer cells. Nano-constructs were prepared by incubating cationic liposomes with siRNA at an optimal condition to form a complex between quaternary nitrogen of DOTAP and phosphate of siRNA molecule. However, nano-constructs formed via mixture of DOTAP and DOPE was very flexible. To provide physical stability and form rigid complex cholesterol and subsequently neutral phosphatidyl choline (HSPC) were used. Gel electrophoresis showed that mixture of DOTAP, DOPE, Cholesterol and HSPC provides stable rigid nano-constructs at N/P charge ratio of 1.5 and above. To ensure complete encapsulation N/P ratio 2.0 was utilized for further applications of nano-constructs. The optimized siRNA

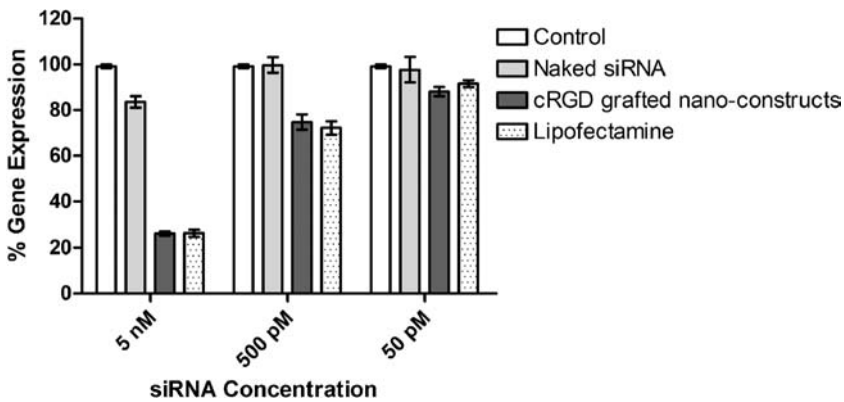


Fig. 10 Transfection studies of developed nano-constructs by Real Time Polymerase Chain Reaction.

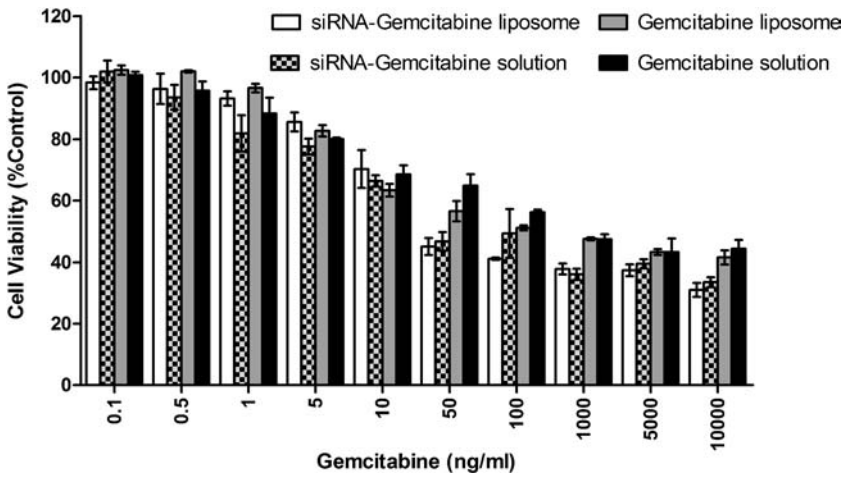


Fig. 11 Chemosensitization in A549 cells with pre-exposure of siRNA nano-constructs.

nano-constructs delivered siRNA more effectively and with lower cytotoxicity than positive control i.e., L2K. PEGylated nanoconstructs after incorporation of cRGD also showed same pattern of complexation. Though N/P 2.0 was utilized for further studies, a firm complex was observed above N/P 1.5.

Lipid composition, size distribution, and surface charge of liposomal system can be easily manipulated to provide a clinically acceptable formulation for systemic delivery. In the process of formation of these nano-constructs, siRNA reacts with the head group of the liposomal DOTAP forming a neutral coordination complex. As a result, nano-constructs bear less charge. The reduction of the membrane surface charge is essential for improving the stability of siRNA-lipid formulations in the blood stream and decreasing the uptake by the reticulo-endothelial system. It has been suggested that the transfection efficiencies of cationic liposomes are affected by several parameters, including the structure and size of the liposomes and their cytotoxicity (22).

siRNA is prone to degrade by nucleases and hence, it is a prime requisite to protect it during circulation in biological fluid. Prepared nano-constructs further provided structural stability to siRNA in presence of FBS, even at 50% v/v concentration. In contrast, naked siRNA degraded by serum nuclease within less than 3 h. Degradation was started within half an hour after incubation of siRNA with FBS. At 0.5 h more than 10% of siRNA was degraded, which reached up to 60% within 3 h. After 6 h only 19.3% of siRNA was remained as compared to initially loaded siRNA. Nano-constructs encapsulated siRNA was stable even after 24 h and more than 90% of siRNA was seen on agarose gel. This stability is a function of complete incorporation of nucleic acid within liposomal structure.

In vitro cell line studies for the cytotoxicity of prepared nano-constructs were thoroughly carried out. From the results of MTT assay, it was seen that at N/P of 2.5 DDCH and cRGD grafted siRNA nano-constructs were significantly ($p<0.05$) less toxic than L2K. Even at higher N/P of 12.5 at 100 nM siRNA concentrations these nano-constructs showed nonsignificant toxicity as compared to L2K. Various reports are available suggesting efficient delivery of siRNA using DOTAP and DOPE but this combination alone is not rigid enough to form physically stable nano-constructs and that may be one of the reasons for low intracellular accumulation of FAM-NC-siRNA as compared to L2K. However, incorporation of cholesterol and HSPC provided sufficient strength to retain siRNA with positively charged liposomal environment. Significant improvement was achieved in MFI values after incorporation of cholesterol and HSPC as compared to other formulations and even more MFI value than L2K suggests the potential of optimized

Table II IC_{50} of Gemcitabine HCl with and without siRNA Pre-treatment

Formulation	IC_{50}
siRNA-Gemcitabine liposome	207.6 ± 1.8
siRNA-Gemcitabine solution	227.6 ± 3.8
Gemcitabine liposome	722.6 ± 11.7
Gemcitabine solution	1088.2 ± 15.7

Values are represented as mean ± SD, $n = 3$

Table III Chemosensitization with siRNA Nano-constructs

Formulations	Fold change in IC_{50}
Gemcitabine solution /siRNA-Gemcitabine liposome	5.241
Gemcitabine solution /siRNA-Gemcitabine solution	4.781
Gemcitabine liposome /siRNA-Gemcitabine liposome	3.480
Gemcitabine liposome /siRNA-Gemcitabine solution	3.174
Gemcitabine solution /Gemcitabine liposome	1.505
siRNA-Gemcitabine solution /siRNA-Gemcitabine liposome	1.091

nano-constructs with complete siRNA encapsulation. Both, quantitative and qualitative, techniques support this hypothesis. Figure shows receptor based localization in case of nano-constructs grafted with cRGD. Live imaging with Z-stack at different time points also confirms that siRNA is not localized to the apical surface of the cells; rather it travelled through the cell membrane inside the cell and thus reveals the targeting potency of cRGD towards cancer cells.

Gene expression studies reveals transfection efficacy of cRGD grafted siRNA nano-constructs targeting RRM1 gene. Transfection at 5 nM strongly down regulates the RRM1 concentration as compared to 500 pM and 50 pM. From the gene expression study it can be seen that 50 pM is sub growth inhibitory concentration. No significant difference was observed at 5 nM concentration for gene expression using L2K and nano-constructs. At lower concentration of RRM1 siRNA i.e., 50 pM the knockdown efficacy was unaffected. Hence, 50 pM was utilized for the chemosensitization studies. The 5-fold increase in Gemcitabine sensitivity following sub-growth inhibitory RRM1 knockdown using siRNA nano-constructs correlates well with a previous report (9), where stably expressed shRNAs were used to knockdown RRM1. Additionally, Gemcitabine liposome showed significantly less IC_{50} value ($P < 0.05$) as compared to Gemcitabine solution in both with and without pre-siRNA treatment. This also demonstrates the application of gemcitabine liposome as a substitute for Gemcitabine solution. Present studies open a vista for chemotherapy at lower dose and at that point it may be possible to formulate clinically suitable liposomes of Gemcitabine HCl with sufficient drug loading. Present studies demonstrate that transfection with as little as 5 nM siRNA caused apoptosis while pM concentrations resulted in a noticeable chemosensitization of the drug Gemcitabine. Taken collectively results suggest that prepared nano-constructs may be a novel therapeutic strategy for reducing a dose of Gemcitabine with combination therapy or alone as a chemotherapeutic agent.

CONCLUSION

Present investigation showed a promising way to treat lung cancer using genomic approach with enhanced margin of safety and reduced dose dependent toxicity of the Gemcitabine HCl. Pre-exposure of cRGD grafted siRNA nano-constructs targeting RRM1 protein caused sensitization of cancer cells. Developed nano-constructs are stable in presence of serum and delivered the siRNA inside the cells along with efficient transfection. Sub-inhibitory concentration of siRNA will avoid toxicity related to localization of siRNA in unwanted sites. Taken collectively, suggested approach will definitely open a vista in the era of cancer treatment with reduced dosing profile.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors acknowledge the financial assistance from Department of Biotechnology (DBT-SBIRI), New Delhi, India and TIFAC CORE in NDDS, Government of India, New Delhi for providing the research facilities to the team. The authors also acknowledge help from Sun Pharmaceuticals, Vadodara for their help in TEM studies.

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