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Chitosan-graft-polyethylenimine for Akt1 siRNA delivery to lung cancer cells

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ABSTRACT

Efficient delivery of small interfering RNA (siRNA) remains a challenging task in RNA interference (RNAi) studies. In this study, we used chitosan-graft-polyethylenimine (CHI-g-PEI) copolymer composed of chitosan and low molecular weight polyethylenimine (PEI) for the delivery of siRNA. The CHI-g-PEI carrier formed stable complexes with siRNA with compact spherical morphology. CHI-g-PEI delivered EGFP siRNA (siGFP) silenced EGFP expression nearly 2.5 folds higher than PEI25K at 50 pM siGFP concentration. Cell viability was found to be 2 folds high with CHI-g-PEI carrier than PEI25K. Also, our CHI-g-PEI carrier efficiently delivered Akt1 siRNA (siAkt) and thereby silenced onco-protein Akt1. Silencing of this crucial cell survival protein significantly reduced the lung cancer cell survival and proliferation. Additionally, Akt1 protein knock-down decreased A549 cell malignancy and metastasis. These findings suggest that the CHI-g-PEI carrier efficiently and safely delivered siRNA. Moreover, CHI-g-PEI mediated Akt1 siRNA delivery may immerge as a viable approach for lung cancer treatment.

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1. Introduction

Discovery of RNA interference (RNAi)-based silencing has drawn much attention to cancer therapy due to the possible targeting of oncogenes. RNAi is a process, whereby the introduction of 21–23 bp long small interfering RNA (siRNA) in cells results in the degradation of homologous mRNA and specific protein knock-down. However, the successful applications of RNAi in therapy depend upon the effective knock-down of targeted transcripts and efficient intracellular delivery of either preformed siRNAs or vector expressed siRNAs (Engelke and Rossi, 2005; Kim et al., 2006).

Chitosan as a polymeric carrier has been successfully applied for *in vitro* and *in vivo* gene delivery applications (Kim et al., 2007; Koping-Hoggard et al., 2001; Ravi Kumar et al., 2004; Lee et al., 2008). Also, a few reports of siRNA delivery using chitosan are available (Rojanarata et al., 2008; Liu et al., 2007; Katas and Alpar, 2006; Howard et al., 2006). Chitosan is a biodegradable, biocompatible, and mucoadhesive cationic polysaccharide with low cytotoxicity, however, its major limitation is low transfection efficiency due to poor buffering capacity for endosomal escape. In previous studies, we have improved the transfection efficiency of chitosan by grafting it with low molecular weight polyethylenimine (PEI) (CHI-g-PEI) (Jiang et al., 2007). This CHI-g-PEI carrier has shown high transfection efficiency with good cell viability *in vitro*, and also *in vivo* on aerosol delivery (Jiang et al., 2007). In this study, we extended the success of CHI-g-PEI carrier for siRNA delivery. Our recent studies on lung cancer showed significant decrease in cancer cell growth on Akt1 siRNA delivery (Jere et al., 2008; Xu et al., 2008). Moreover, aerosol delivered Akt1 siRNA suppressed lung tumorigenesis in K-ras^{LA1} and urethane-induced tumor mice models (Xu et al., 2008). On this background, we evaluated the efficiency of CHI-g-PEI carrier in Akt1 siRNA (siAkt) delivery to A549 cells due to the known benefits of chitosan on *in vivo* aerosol application (Jiang et al., 2007). CHI-g-PEI–siRNA system was used for the silencing of reporter protein EGFP (enhanced green fluorescence) and oncoprotein Akt1. Also, the consequences of Akt1 silencing on cancer cell survival, proliferation, malignancy and metastasis were evaluated *in vitro* (Fig. 1).

2. Materials and methods

2.1. Materials

Chitosan (molecular weight 100 kDa; deacetylation degree 87.7%) was kindly supplied from Jakwang (Ansung, Korea). Branched PEI25K, and potassium periodate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Branched PEI 1.8 kDa was purchased from Wako (Osaka, Japan). Plasmid pEGFP-N2, with early

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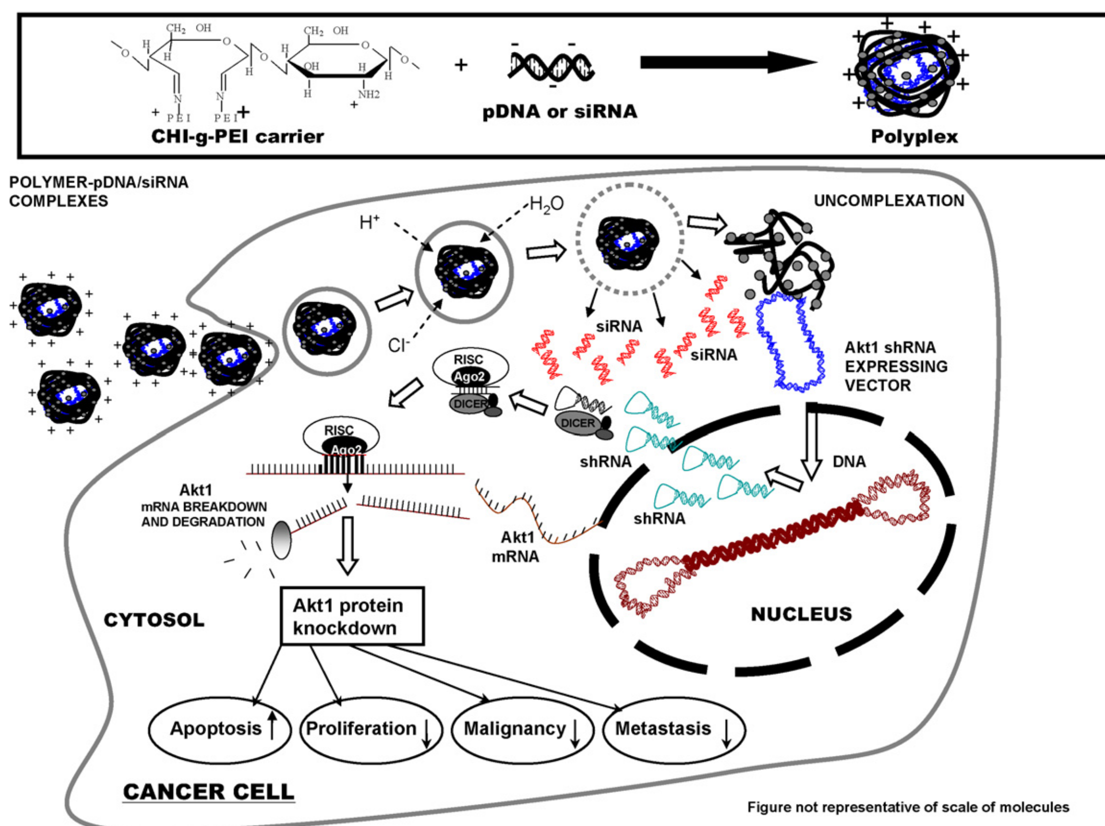


Fig. 1. Schematic representation of oncogene Akt1-targeted shRNA and siRNA delivery to cancer cell using CHI-g-PEI carrier and its consequences on cancer cells survival.

CMV promoter and EGFP gene, was obtained from Clontech (Palo Alto, CA, USA). *Silencer*™ GFP (cat: 4626) siRNA specific for EGFP (siGFP) and scrambled siRNA (scrsiRNA) were purchased from Ambion (Austin, TX, USA). The targeted Akt1 mRNA sequence is GAAGGAAGUCAUCGUGGCCAA (Meng et al., 2006; Jere et al., 2008). Akt1 siRNA with proprietary modifications to improve stability and specificity were synthesized from Dharmacon (Chicago, IL, USA). Also, the cassette of oligonucleotides encoding 19-mer hairpin sequences specific to the same target sequence was designed, the sense and antisense strands were synthesized, annealed and ligated into the linearized pSilencer 2.0-U6 vector (Mirus, USA). A scrsiRNA with the same nucleotide composition as the siRNA but which lacks significant sequence homology to the genome was also designed. Antibody for Akt1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primers were designed and synthesized from Bioneer (Seoul, Korea). Cell-to-cDNA™ kit was purchased from Ambion Inc. (Austin, TX, USA). Rhodamine-labeled siGLO™ RISC free siRNA was purchased from Dharmacon (Chicago, IL, USA). Annexin V-FITC and propidium iodide kit was purchased from Abcam (Cambridge, UK).

2.2. Synthesis and characterization of CHI-g-PEI

Synthesis of CHI-g-PEI was carried out by imine reaction between periodate-oxidized chitosan and low molecular weight PEI (1.8 kDa) exactly as described in our previous paper (Jiang et al., 2007). Briefly, chitosan 100K (0.1 M) and potassium periodate (0.01 M) were dissolved in sodium acetate buffer (pH 4.5), degassed with N_2 and adjusted to 4 °C. Reaction was carried out for 48 h and stopped by adding ethylene glycol (10% v/v) then dialyzed against NaCl (0.2 M, pH 4.5) and deionized water (pH 4.5). In the second step PEI 1.8 kDa (20 mM) was reacted with the periodate-oxidized chitosan solution (10 mM) with stirring for 2 days at 4 °C. Subsequently treated with sodium borohydride (2 g) and dialyzed against NaCl (0.2 M, pH 4.5) and deionized water at 4 °C followed by lyophilization. The reaction scheme is shown in Fig. 2. The confirmation of synthesis and the composition of the prepared CHI-g-PEI were determined by ^{1}H nuclear magnetic resonance (^{1}H NMR) (Advance™ 600, Bruker, Germany). The molecular weight of CHI-g-PEI copolymer was measured by gel permeation chromatography column with multi-angle laser scattering (GPC-MALS) at 690 nm laser wavelength (Dawn Eos, Wyatt, USA).

2.3. Complex formation

For a complex formation study, the siRNA solution was added to the CHI-g-PEI (N/P = 35) solution to make final volume of 15 μl with siRNA concentrations 37, 75 and 150 pmol and vortex-mixed gently. After 30 min incubation complexes were loaded on 3% agarose gel and subjected to electrophoresis for 30 min at 50 V. The gel was analyzed on UV illuminator after 30 min of EtBr (0.5 mg/ml) soaking.

2.4. Morphology and size of complexes between CHI-g-PEI and siRNA

The CHI-g-PEI–siRNA complexes were prepared at 100 pmol siRNA concentration. One drop of CHI-g-PEI–siRNA complexes were placed on a copper grid and stained with 2% PTN solution for 10 s. The complex morphologies were observed using transmission electron microscopy (TEM) (LIBRA 120, Carl Zeiss, Germany).

2.5. Transfection of CHI-g-PEI–siGFP complexes

A549 cells (ATCC) (2 × 10^5 cells/well) were seeded in a 12 wells plate (SPL Life Sciences) and allowed to attach overnight in RPMI

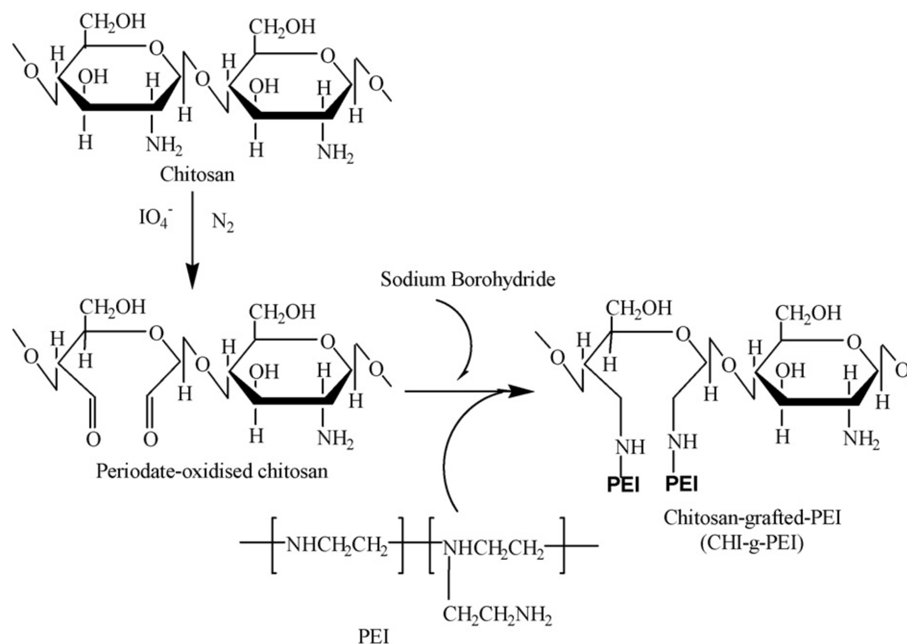


Fig. 2. Reaction scheme for synthesis of CHI-g-PEI.

1640 medium (10% FBS) (Hyclone, South Logan, UT). Complexes between Lipofectamine™ and plasmid pEGFP-N2 were prepared as per the manufacture's protocol, and cells were transfected for expressing stable EGFP protein. For silencing study, complexes between CHI-g-PEI (N/P = 35) and siGFP/scrsiRNA were prepared at 50 pmol siRNA concentration in the presence of nuclease free sterile sodium acetate buffer (25 mmol; pH 5.5) by incubating for 30 min. Similarly, complexes were prepared between PEI25K and siGFP/scrsiRNA (N/P = 10). Final volume was adjusted to 1 ml by adding serum free media. The complexes were transferred to each well of 12 wells plate and incubated for 12 h, and then the media were changed with fresh media containing serum and incubated at 37 °C and 5% CO_2 for 48 h. After 48 h incubation the efficiency of silencing was measured by FACS and confocal microscopy. The percent EGFP silencing was calculated after normalizing the results with mock and scrambled treated cells. All experiments were performed in triplicates.

2.6. Transfection of CHI-g-PEI–siAkt complexes

A549 cells (2×10^5 cells/well) were seeded in a 6 wells plate (SPL Life Sciences) and allowed to attach overnight in RPMI 1640 media (10% FBS), 100 U/ml penicillin. Complexes were prepared between CHI-g-PEI and Akt1 siRNA (siAkt) or Akt1 shRNA expressing plasmid (shAkt) (2 μg/ml) at N/P ratio 35:1 in a nuclease free sterile sodium acetate buffer (25 mM; pH 5.5) and water by incubating for 30 min. Similarly, complexes were prepared between scrsiRNA and CHI-g-PEI. In case of PEI25K, transfections were performed at N/P ratio10. After complexes formation, the final volume was adjusted to 2 ml by adding serum free media. The complexes were transferred and incubated for 12 h, and then the media was changed with fresh media containing serum and incubated at 37 °C and 5% CO_2 for 24, 48, or 72 h depending on the subsequent experiments. Blank, mock carrier, scrsiRNA, and PEI25K were used as controls.

2.7. Uptake of CHI-g-PEI–siRNA complexes

The complexes between CHI-g-PEI and rhodamine labeled-siRNA were prepared as described above and incubated with cells for 3 h. After incubation, cells were washed repeatedly with ice cold PBS and fixed by using 10% formaldehyde. Post-transfection images were taken by confocal laser scanning microscopy (CLSM) at 597 nm wavelength.

2.8. Western blot analysis

After 48 h transfection with siAkt and shAkt, cells were lysed for total protein extraction and quantified by BCA protein assay kit (Promega, USA). Equal amount of protein was separated on SDS-PAGE and transferred onto nitrocellulose membrane. Then the membrane was blocked and incubated overnight with Akt1 (c20) goat polyclonal antibody. After washing, the membrane was incubated with horseradish peroxidase-labeled secondary antibody. The bands of desired proteins were visualized using the Westzol enhanced chemiluminescence kit (Intron, Sungnam, Korea). The Akt1 expression was normalized with house-keeping gene expression.

2.9. Reverse transcription-PCR

Twenty four hours post-transfection with siAkt and shAkt, total mRNA was extracted from 1×10^6 cells and reverse transcribed into cDNA by using Cell-to-cDNA™ kit as per manufacture's protocol. The amplification and quantification were performed exactly as reported in our previous paper (Jere et al., 2008).

2.10. Cell viability assays for CHI-g-PEI–shAkt complexes

MTS and trypan blue dye exclusion assays were performed for A549 cells treated with mock carriers, shAkt and scrsiRNA using CHI-g-PEI and PEI25K carriers as described earlier (Jere et al., 2008).

2.11. Apoptosis measurement

A549 cells were transfected and 72 h post-transfection, floating and adherent cells (1×10^5) were collected and stained with Annexin V-FITC and PI according to the manufacturer's instruc-

tions. Cells undergoing apoptosis and necrosis were determined by FACS. The cells under going apoptosis and necrosis were normalized with control and percentage was calculated. Experiments were performed in triplicates.

2.12. Cell proliferation assay

shAkt and scrsiRNA treated cells and untreated A549 cells were seeded (2×10^5 cells/well) in RPMI 1640 medium (10% FBS) and

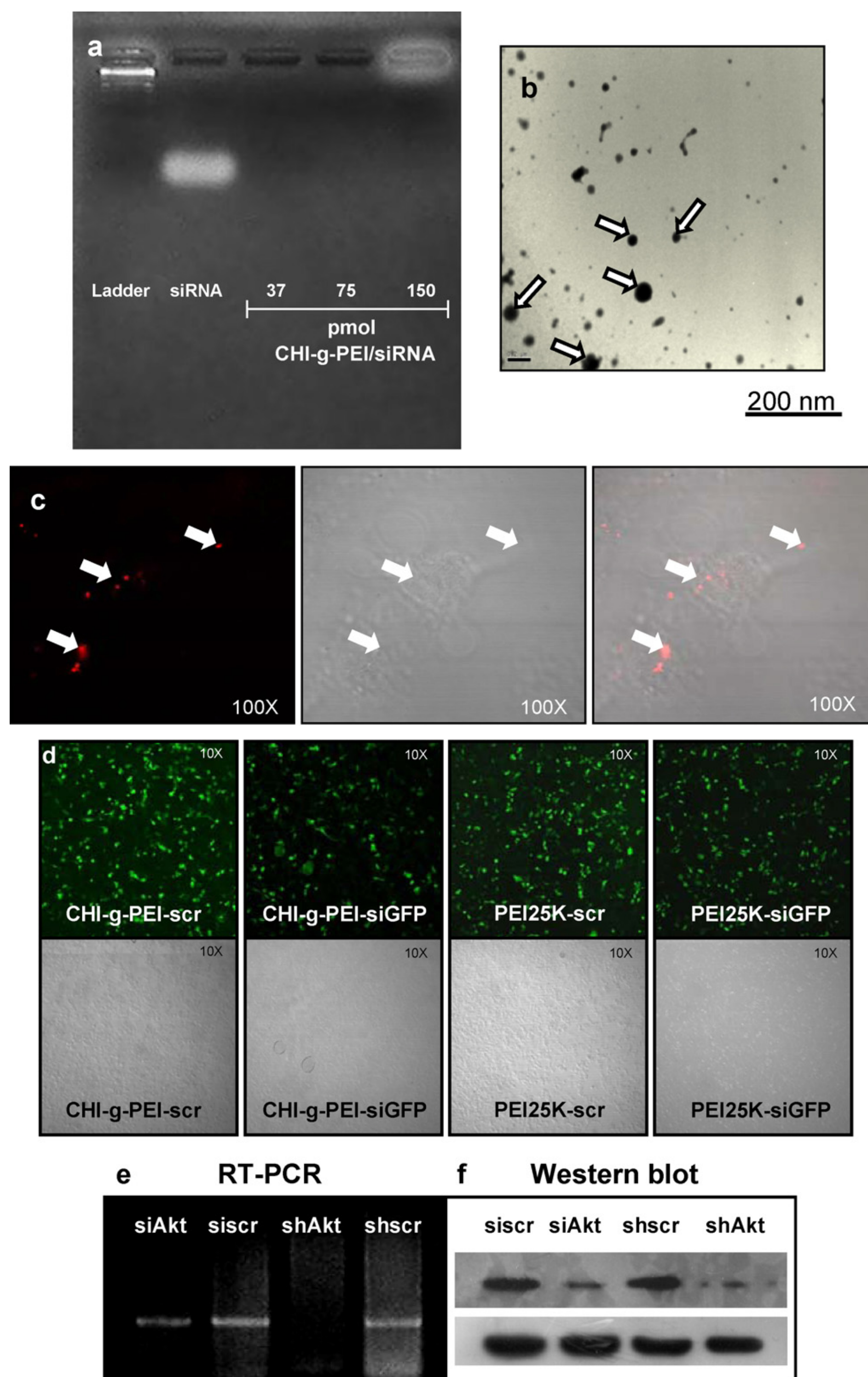


Fig. 3. Complexation and condensation of siRNAs by CHI-g-PEI carrier: (a) agarose gel electrophoresis of CHI-g-PEI–siRNA complexes at different siRNA concentrations (37, 75 and 150 pmol), (b) morphology of CHI-g-PEI–siRNA complexes (siRNA concentration 100 pmol) by transmission electron microscope (scale bar: 200 nm), (c) intracellular tracking of Rhodamine labeled CHI-g-PEI–siRNA complexes by confocal laser scanning microscopy (magnification 400×): dark field image (emission wave length 597 nm) (left image), phase contrast image (central image) and image obtained after overlapping dark field and phase contrast images (right image), (d) confocal laser scanning microscopic images of siGFP and scrsiRNA treated cells using CHI-g-PEI and PEI25K carriers, dark field images (upper images), phase contrast images (lower images), (e) semiquantitative RT-PCR analysis for the confirmation of Akt1 mRNA knock-down and (f) Western blot analysis for the confirmation of the silencing of Akt1 protein expression post-shAkt and siAkt treatments using CHI-g-PEI carrier.

allowed to proliferate at 37 °C in 5% CO_2 for 24, 48, and 72 h. Cells were harvested and counted at different time intervals. The total cell proliferation at different time intervals was determined after normalizing with the scrsiRNA treated cells at 0 h. Assay was performed in triplicates.

2.13. Soft agar assay

After 48 h shAkt and scrsiRNA transfected cells (1×10^5) were trypsinized, washed and resuspended in medium containing 0.3% low-melting agarose at 37 °C, which was layered on top of solidified 0.6% agarose bottom layer in 6 wells plates in triplicates. Assay was performed in RPMI 1640 media (10% FBS) layered on top of agarose, and media was changed every alternate day. After 15 days incubation at 37 °C in 5% CO_2, the total numbers of colonies were counted after staining with *p*-iodonitrotetrazolium violet (Sigma–Aldrich, USA) and images were captured.

2.14. Cell migration assay

In the migration assay, 48 h post-transfection with shAkt (1×10^5) cells were seeded in the upper chamber of a 6 well transwell chamber in serum free media. RPMI 1640 medium with 20% serum was used as a chemo-attractant in the lower chamber. Cells were allowed to migrate for 24 h at 37 °C in 5% CO_2, then cells on the upper surface of the membrane were removed mechanically. Migrated cells were fixed with 100% ice cold methanol, stained with Giemsa (Sigma, USA) (1:25 in PBS) and observed under microscope. Total migrated cells in each well were counted in three different fields per experiment by using Neubauer chamber and photographed.

3. Results

3.1. Synthesis and characterization of CHI-g-PEI

Periodate-oxidized chitosan was successfully grafted with branched PEI (1.8 kDa) by an imine reaction as shown in Fig. 2. ^{1}H NMR confirmed the synthesis of CHI-g-PEI carrier and molecular weight of CHI-g-PEI was around 25 kDa as determined by GPC-MALS analysis. Although, chitosan is soluble only in acidic conditions, after grafting PEI, CHI-g-PEI became completely water soluble at physiological pH due to the hydrophilic nature of PEI. The comprehensive characterization of CHI-g-PEI and its application in gene delivery has been reported by us earlier (Jiang et al., 2007).

3.2. Evaluation of CHI-g-PEI for siRNA delivery

Stable nano-size complex is a prime requirement in siRNA delivery. In compliance, CHI-g-PEI formed stable and compact complexes with siRNAs. Gel retardation assay performed to evaluate siRNA binding capacity of CHI-g-PEI showed efficient complex formation between CHI-g-PEI and siRNAs at different concentrations. CHI-g-PEI carrier was able to complex as high as 150 pmol siRNA without leaching (Fig. 3a). Also, TEM imaging revealed small, compact and spherical morphologies of the complexes with particle sizes smaller than 150 nm (Fig. 3b). Post-transfectional CLSM imaging of rhodamine labeled-CHI-g-PEI–siRNA complexes further substantiated the efficient intracellular uptake and distribution of siRNAs (Fig. 3c).

Finally, CHI-g-PEI delivered anti-EGFP siRNAs (siGFP)s silenced EGFP expression approximately 2.5 folds higher than that of PEI25K in A549 cells. In flow cytometry analysis, around 55 and 19% silencing were observed on siGFP delivery using CHI-g-PEI and PEI25K carriers, respectively. Also, confocal microscopic observation of EGFP knock-down re-affirmed the flow cytometric results, and confirmed the superiority of CHI-g-PEI carrier over PEI25K (Fig. 3d).

After successful EGFP siRNA delivery, CHI-g-PEI carrier was evaluated for the delivery of therapeutic siRNA to target Akt1 protein whose over-expression is mainly responsible for cancer cell survival, proliferation, and metastasis (Datta et al., 1999; Brognard et al., 2001). In our previous reports, we have discussed in detail the vital role of Akt1 protein in lung cancer, and the effects of its silencing on lung cancer progression (Jere et al., 2008; Xu et al., 2008). CHI-g-PEI-mediated siAkt and shAkt delivery efficiently knocked-down Akt1 mRNA as confirmed from RT-PCR (Fig. 3e). Western blot analysis further confirmed the decrease in Akt1 protein expression as the consequence of Akt1 mRNA knock-down (Fig. 3f).

3.3. Cell viability

Safety is a prime obligation on si/shRNA delivery system for clinical application. CHI-g-PEI carrier has already been reported to be safe for gene delivery applications (Jiang et al., 2007). In accord, CHI-g-PEI carrier has displayed high safety for siRNA delivery applications. In MTS assay, approximately 90% cell viability was observed for CHI-g-PEI and CHI-g-PEI–scrsiRNA, on contrary, only 50% cells were survived with mock PEI25K and PEI25K-scrsiRNA treatments (Fig. 4a). The differences in the cell viability with mock and scrsiRNA treatments were negligible. The trypan blue dye exclusion assay further reaffirmed the MTS results, and was exhibited 85 and 50% cell viability with CHI-g-PEI and PEI25K treatments, respectively (Fig. 4b). Additionally, CHI-g-PEI–shAkt and PEI25K-shAkt delivery reduced the cell number by approximately 35 and 20%, respectively. This post-transfectional decrease in cell number is a cumulative effect of carrier associated cytotoxicity, Akt1 siRNA mediated reduced proliferation and induced apoptosis. Collectively,

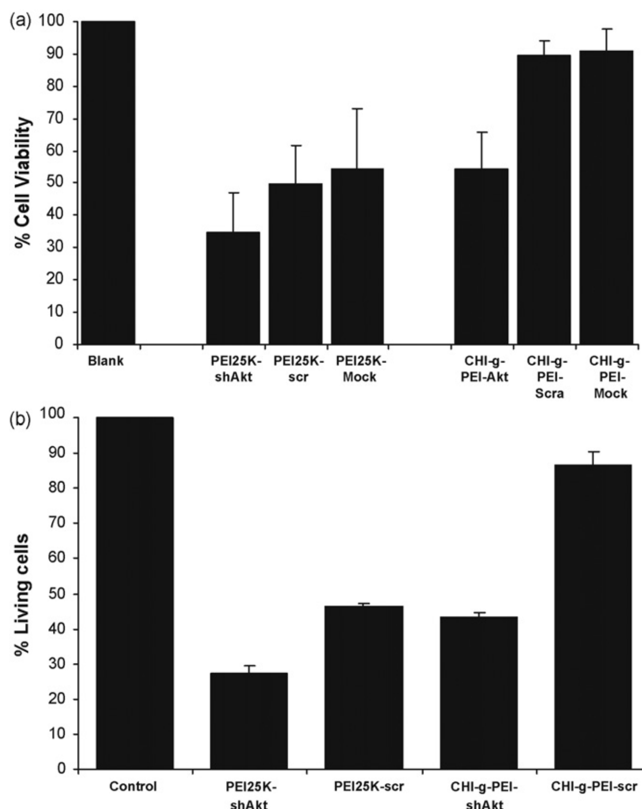


Fig. 4. Cell viability assay for measuring A549 cell number reduction after CHI-g-PEI and PEI25K treatments (a) MTS assay and (b) trypan blue dye exclusion assay ($n = 3$, error bar represents standard deviation).

these assays indicate better safety of CHI-g-PEI carrier over the standard PEI25K.

3.4. Cancer cell proliferation and apoptosis

Unrestricted proliferation of cancer cells enfeebles on post-Akt1 knock-down (Heron-Milhavet et al., 2006; Meng et al., 2006). Moreover, it induces apoptosis in cancer cells (Jere et al., 2008; Meng et al., 2006; Sithanandam et al., 2005). However, the magnitudes of these effects depend upon the delivery efficiency of shAkt which further executes Akt1 silencing. CHI-g-PEI mediated shAkt delivery efficiently reduced A549 proliferation and growth progression. The reduction in cell proliferation started post-24 h of transfection. After 48 h transfection, the reduction in cell proliferation was 1.6 folds with CHI-g-PEI–shAkt treatment. Moreover, 72 h post-transfection, the peak reduction of 4 folds was observed. At all time points, reduction in cell proliferation was significantly higher with CHI-g-PEI–shAkt than PEI25K-shAkt treatment. After 72 h, the decrease in cell proliferation with CHI-g-PEI–shAkt was 2 folds higher than PEI25K-shAkt as shown in Fig. 5a. In addition to decreased proliferation, Akt1 silencing also induced apoptosis in A549 cells. Flow cytometric analysis with Annexin V-FITC and PI labeling revealed approximately 20% increase in apoptosis with CHI-g-PEI–shAkt treatment which was 5 folds higher than that of control. While, PEI25K showed nearly 4 folds increase in Akt1 specific apoptosis (Fig. 5b). These results indicate good shAkt delivery efficiency of CHI-g-PEI carrier in cancer studies.

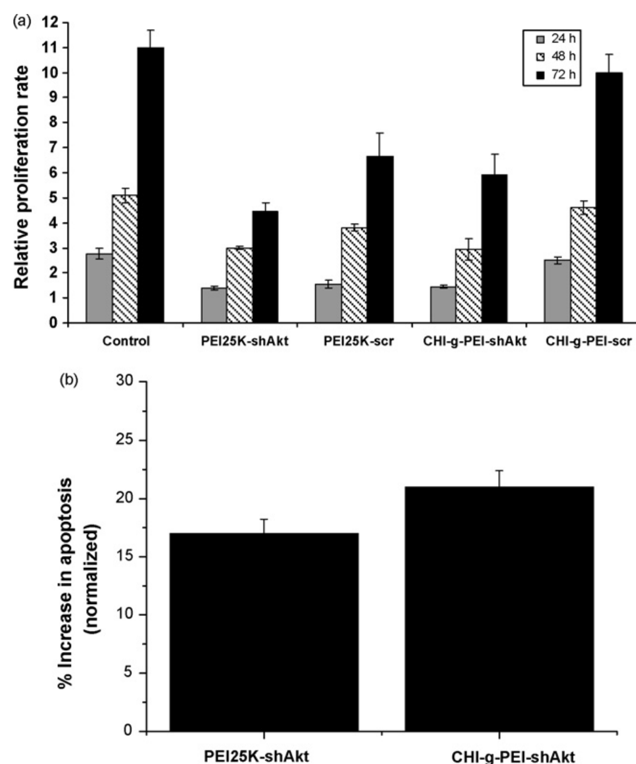
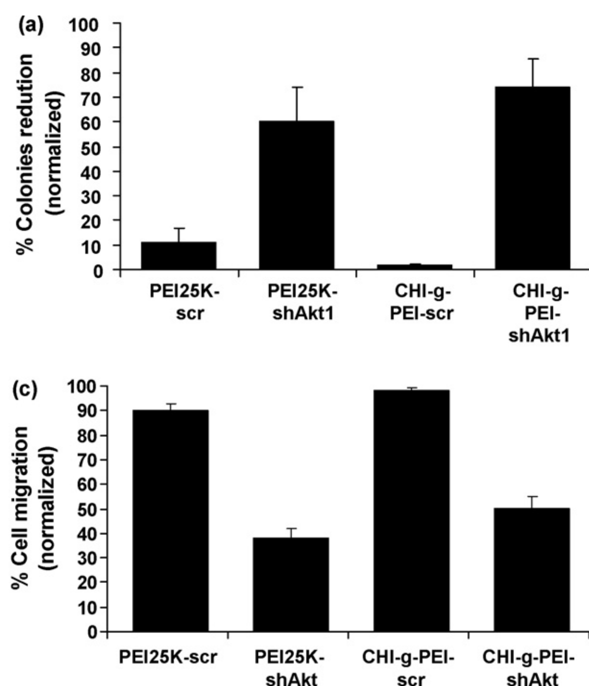


Fig. 5. (a) Cell proliferation of shAkt treated A549 cells using CHI-g-PEI carrier at different time interval (normalized with shscr treatments at 0 h) ($n = 3$, error bar represents standard deviation) and (b) percent increased in apoptosis due to shAkt treatment with CHI-g-PEI carrier (values normalized with control and shscr treatments) ($n = 3$, error bar represents standard deviation).

3.5. Cancer cell malignancy and metastasis

Currently, cancers are incurable because of the malignant and metastatic growth potentials of the cancer cells. *Anchorage independent* growth exhibited by malignant cells approbates metastatic spread of the cancer cells. Soft agar assay is a specific method wherein malignant cancer cells grow and form distinguishable floating colonies on soft agar gel. In this assay, we observed significant reduction in A549 soft agar colonies on CHI-g-PEI–shAkt treatment (Fig. 6a). CHI-g-PEI–shAkt showed approximately 70% reduction while PEI25K-shAkt showed approximately 50% reduction as compared to scrambled treatment. Moreover, CHI-g-PEI–shAkt treatment reduced the migration of



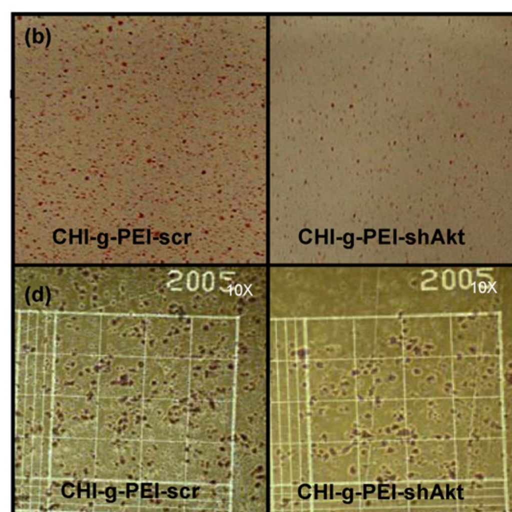


Fig. 6. (a) Percentage of colony reduction relative to scrambled-transfected cells in soft agar assay, (b) inhibition of A549 growth due to shAkt treatment by CHI-g-PEI carrier on soft agar, (c) percent cell migration of Akt knocked-downed A549 cells using CHI-g-PEI and PEI25K carrier (normalized with blank) and (d) migration in transwell assay observed under optical microscope (magnification 100×).

A549 cells in transwell assay. CHI-g-PEI–shAkt and PEI25K-shAkt treatments showed approximately 50% reduction in migratory tendency (Fig. 6b). These results clearly indicate the significance of Akt1 siRNA treatment in oncoprotein Akt1 silencing using CHI-g-PEI carrier in lung cancer studies.

4. Discussion

Chitosan is a biocompatible polysaccharide most extensively studied for gene and siRNA delivery, mainly due to its cationic nature and mucoadhesive property. Number of modifications of chitosan has been proposed for gene and/or siRNA delivery (Kim et al., 2007). Amongst them, the CHI-g-PEI carrier constituting chitosan and branched PEI has immerged as a potential candidate for siRNA delivery due to its remarkable *in vitro* and *in vivo* gene delivery success (Jiang et al., 2007). As a primary requirement of efficient siRNA delivery, CHI-g-PEI must form stable complex with siRNA as it does with pDNA. However, complexing siRNA is more challenging due to its stiff molecular topology and limited anionic charge (Bolcato-Bellemin et al., 2007). siRNAs with non-optimal condensation are vulnerable to leaching and degradation (Bolcato-Bellemin et al., 2007). Also, improper condensation results into large non-spherical complexes which can be rapidly cleared from the body. CHI-g-PEI displayed good siRNA condensing and complexing ability without any leaching and fulfilled the prime requirement in siRNA delivery. Also, it formed small, compact and spherical complexes for rapid uptake by cancer cells. These complexes showed efficient cellular entry and high perinuclear distribution as observed from the cytosolic fluorescence of rhodamine-labeled complexes. Moreover, CHI-g-PEI mediated siGFP delivery reduced EGFP fluorescence 2.5 times higher than that of PEI25K substantiating high siRNA transfection efficiency of CHI-g-PEI carrier. All these results collectively suggested good siRNA delivery potential of CHI-g-PEI carrier in RNAi studies.

In RNAi studies, especially for cancer, oncoprotein targeting is a promising approach, and can be accomplished by oncogene specific siRNA delivery. As our CHI-g-PEI carrier exhibited good siRNA delivery efficiency, we decided to evaluate it for the delivery of anti-oncoprotein siRNA. Akt1 is a prime cell survival protein overexpressed in lung and numerous cancers (Sourbier et al., 2006; Sithanandam et al., 2005; Staal, 1987). Our recent studies on lung cancer have established the importance of Akt1 silencing in restricting lung tumorigenesis and proliferation *in vitro* and *in vivo* (Jere et al., 2008; Xu et al., 2008). In harmony, CHI-g-PEI carrier delivered siAkt and shAkt efficiently reduced Akt1 protein expression in A549 cells. RT-PCR analysis further validated that the decrease in Akt1 protein expression was the consequence of reduced Akt1 mRNA levels. CHI-g-PEI-mediated shAkt delivery was found to be safe and specific as indicated by cell viability assays. These assays displayed high safety of CHI-g-PEI carrier over PEI25K on scrambled siRNA treatment. Also, they displayed 1.5 times reduction in A549 cell number after CHI-g-PEI–shAkt treatment and provided a preliminary indication of induced apoptosis and reduced proliferation post-Akt1 silencing. Further, annexin V-FITC and PI labeling assay confirmed the increase in apoptosis on Akt1 siRNA treatment as compared to control. Furthermore, proliferation of A549 cells reduced, and this reduction in proliferation was continued over the 72 h time period, indicating the important role of Akt1 protein in cancer cell proliferation. In accord with previous findings, Akt1 silencing decreased the malignant growth capacity on soft agar. Also, it reduced the migration of A549 cells in transwell assay.

Above findings suggest that our biocompatible CHI-g-PEI carrier composed of chitosan and branched PEI has a great potential in siRNA-based cancer studies. CHI-g-PEI efficiently and safely delivered siRNA in lung cancer cells. CHI-g-PEI delivered siAkt and shAkt silenced Akt1 protein efficiently which hampered lung cancer cell survival in Akt1 specific manner. Also, in accord with previous findings, Akt1 protein knock-down reduced A549 cell survival, proliferation, malignancy and metastasis *in vitro*. Thus, CHI-g-PEI carrier is efficient and safe in siRNA delivery and can be successfully employed in sh/siRNA-based cancer studies.

Acknowledgements

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