Department of Pharmaceutical Biotechnology[1], Faculty of Pharmacy; Pathology Laboratory[2], Vocational Health School, Marmara University, Istanbul, Turkey

# *In vitro* silencing effect of chitosan nanoplexes containing siRNA expressing vector targeting VEGF in breast cancer cell lines

E. Şalva[1,2], J. Akbuğa[1]

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*Dr. Emine Salva, Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Tıbbiye Cad. Haydarpaşa, 34160 Istanbul, Turkey*
*emine_salva@yahoo.com*

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Small interferring RNA (siRNA) is a powerful tool for controlling cellular processes of gene silencing at post-transcriptional level due to its high sequence-specific inhibition efficiency. The aim of this study is to compare silencing effect of chitosan/shVEGF (shRNA-expressing plasmid DNA targeting vascular endothelial growth factor-A (VEGF-A)) nanoplexes between four different cell lines, two of which are MCF-7 and MDA-MB435 breast cancer cell lines. Nanoplexes were prepared using different concentrations. The morphological and physicochemical characterization were made and silencing activity of appropriate formulations were investigated *in vitro*. As a result of *in vitro* transfection studies made in different cell lines with nanoplexes in the different ratios, the highest gene inhibition (60%) was measured in MCF-7 after transfection while the lowest gene inhibition (29%) was observed in MDA-MB435. This work suggests that RNA interference has potential to be applied to delivery system studies and to the angiogenesis treatment.

## 1. Introduction

Inhibition of angiogenesis growth factors and administration of angiogenesis inhibitors are the basics of antiangiogenesis therapy. Vascular endothelial growth factor (VEGF) is the major growth factor that stimulates *in vitro*/*in vivo* angiogenesis, therefore today VEGF is used as a new target for cancer treatment (Ferrara et al. 2005). The discovery of RNA interference (RNAi) mediated gene silencing strategy has introduced a promising dimension to the area of gene therapy research. RNAi technology, a sequence specific silencing of genes, is induced by small molecules of dsRNA, thus blocking the translation of the mRNA into protein (Elbashir et al. 2001; Hannon 2002; Tan et al. 2007). Generally, there are two strategies to introduce siRNA into mammalian cells: i) chemical synthesis strategy in which the synthetic siRNA is directly introduced into target cells or ii) DNA-based (plasmid or viral vector) strategy in which the siRNA is produced via intracellular processing of the expressed RNA hairpin transcript (Lu and Mahato 2009).

Although siRNA can be potential therapeutic agents for various diseases including cancer, the problem of delivery or appropriate delivery system is a major obstacle to the clinical use of RNAi based drugs (Leung et al. 2005; Takeshita et al. 2006). This silencing technology has been successfully applied to inhibit target gene expression, but its utility is limited by its short half-life. Effective therapeutic *in vitro* or *in vivo* gene silencing in mammalian cells and tissues would require DNA-based shRNA strategy rather than transient transfection of double-stranded RNA (Yoo et al. 2007). Typically, synthetic siRNA mediates silencing effects for 2-3 days post-transfection, while the shRNA expression vector produces siRNAs continously in cells and silence the expression of target gene for weeks or even months (Lu and Mahato 2009). Therefore, we constructed a short hairpin RNA (shRNA)-expressing pDNA system (shVEGF) targeting vascular endothelial growth factor-A (VEGF-A), a key mediator in angiogenesis.

One means to achieve this longlasting expression of siRNA is to use a vector-based delivery system such as non-viral vectors. However, the success of siRNA treatment is largely dependent on the delivery vehicle which should be administered efficiently, safely and repeatedly (Zhang et al. 2007). Among non-viral vectors, chitosan has been considered to be a good gene carrier since it is known as a biodegredable, biocompatible and low toxic biopolymer with high cationic potential (Akbuga et al. 2004; Borchard 2001; Kim et al. 2007; Ozbas et al. 2003). Although chitosan has been widely investigated as a gene vector for pDNA, there is a very limited number of studies that have been carried out to investigate the use of chitosan for siRNA delivery (Liu et al. 2007; Salva et al. 2008). Howard et al. (2006) have developed a novel chitosan-based siRNA nanoparticle delivery system for RNA interference *in vitro* and *in vivo*. They showed nanoparticle-mediated knockdown of enhanced green fluorescent protein (EGFP) in human lung carcinoma cells (H1299) and murine peritoneal macrophages (77.9% and 89.3% reduction, respectively). Effective *in vivo* results were also obtained after nasal application of chitosan/siRNA formulation to transgenic EGFP mice (Howard et al. 2006). The same group studied the influence of polymeric properties of chitosan on chitosan/siRNA nanoparticles and gene silencing. Their studies showed that the physicochemical properties and *in vitro* silencing effect of nanoparticles are strongly dependent on chitosan properties (mol wt and degree of deacetylation) (Liu et al. 2007). Chitosan-thiamine pyrophosphate nanoparticles were also developed as a novel carrier for siRNA delivery (Rojanarata et al. 2008). Jere et al. (2009) studied chitosan-graft-PEI for the delivery of Akt1

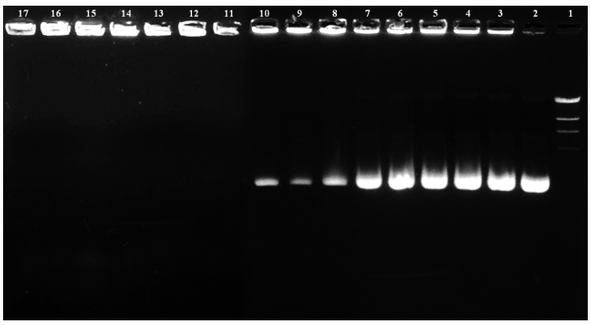



Fig. 1: Agarose gel electrophoresis of chitosan/shVEGF nanoplexes at various N/P ratios
1.Standard DNA (λDNA/HindIII), 2.shRNA-VEGF, 3-17.chitosan/shRNA (+/-) nanoplexes (0.05/1, 0.07/1, 0.08/1, 0.09/1, 0.1/1, 0.2/1, 0.3/1, 0.4/1, 0.5/1, 1/1, 2/1, 4/1, 6/1, 8/1 and 10/1)

siRNA to lung cancer cells and 2.5 folds higher silencing effect was obtained.

There are several gene inhibition studies with VEGF-targeted shRNA in different tumor models (Bai et al. 2009; Jiang et al. 2005; Shibata et al. 2008). On the other hand, several delivery systems such as cationized gelatin (Matsumoto et al. 2006), atelocollogen (Takei et al. 2004), PLGA (Murata et al. 2008) and polyelectrolyte complex micelles (Al-Abd et al. 2009) have been used to improve intracellular delivery of VEGF siRNA, but no study is available on VEGF shRNA-chitosan nanoplex systems. In recent studies, cell-dependent transfection has been reported for different carriers and a correlation was demonstrated between gene expression and cellular uptake (Ishii et al. 2001).

The aim of this study was to examine the silencing effect of chitosan/shVEGF nanoplexes in different cell lines for further *in vivo* studies.

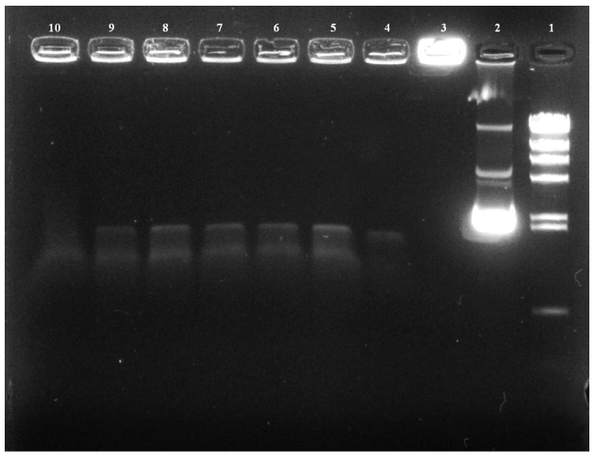
## 2. Investigations, results and discussion

Vector-based RNAi is another strategy to induce RNAi effects with a therapeutic potential. In this study, preparing chitosan/shRNA nanoplexes, we investigated the potential of chitosan to be carrier system for VEGF siRNA. At the same time, this study also emphasizes the potential of RNAi exploitation in breast cancer therapy. VEGF is an antiangiogenic factor that plays an important role in sustaining the growth of many solid tumors and thus it represents an ideal target for an antiangiogenic therapy (Niola et al. 2006).

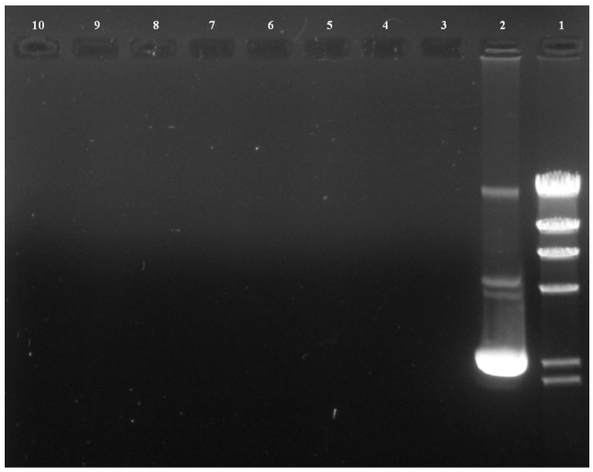
In this study, chitosan/shRNA nanoplexes were prepared for VEGF inhibition based RNA interference method and *in vitro* silencing effects of nanoplexes were studied in different cell lines particularly in breast cancer cell lines. In addition, importance of the selection of cell line in *in vitro* silencing studies was emphasized.

### *2.1. Preparation and control of chitosan/shRNA nanoplexes*

To identify the formation of chitosan/shRNA nanoplexes, a gel retardation assay at different w/w ratios was performed. Different ratios of chitosan to shRNA were tested at fixed shRNA concentration (from 0.05/1 to 10/1 ratio). Full complexation was observed after chitosan/shRNA in 0.5/1 ratio (Fig. 1). Below this ratio, partly complexation formed between chitosan and shRNA. As shown in Fig. 1, when the value of +/- ratio of chitosan/shRNA reached to 0.5/1, free shRNA could not be detected on agarose gel.




(a)



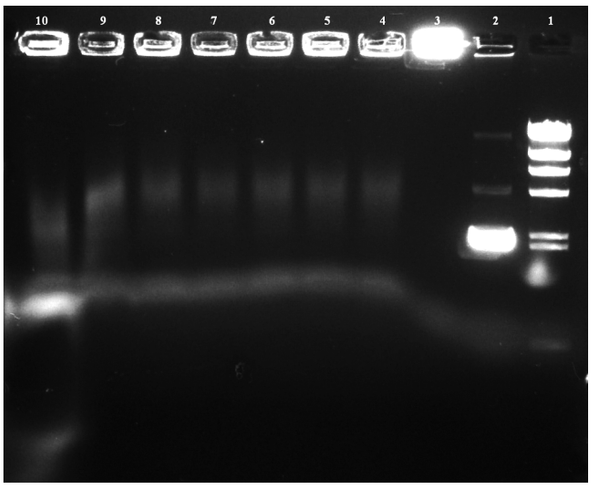

(b)

Fig. 2: **a:** Enzyme stability of chitosan/shVEGF nanoplex
1.Standard DNA(λDNA/HindIII), 2.shVEGF, 3.Blank (chitosan/shVEGF;2/1) 4-10. 0,5,15,30 min, 1,4,24 h
**2b:** Enzyme stability of free shVEGF
1.Standard DNA (λDNA/HindIII), 2.shVEGF, 3-10. 0,5,10,15, 30,60 min., 2,4 h

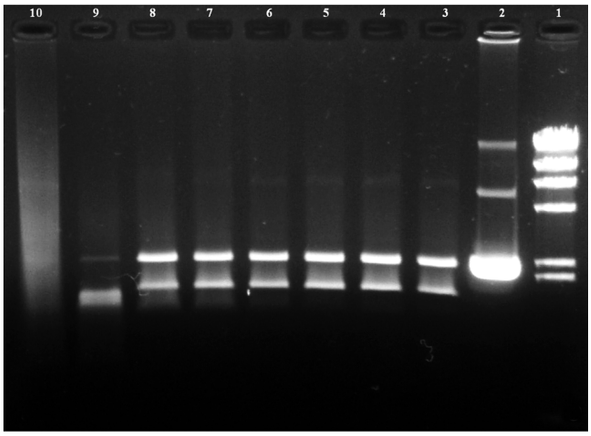
### *2.2. Stability of shRNA in serum and enzyme*

Enzyme and serum stabilities of nanoplexes are necessary for *in vivo* and *in vitro* efficiency of shRNA. However, it is important that shRNA dissociate from chitosan complex inside the cell for its interaction with intracellular components at RISC in RNAi-mediated gene silencing process. Stable chitosan/shRNA nanoplexes are important in terms of protection of DNA in the endosomal compartments, but this situation restricts the introduction of the DNA to the nucleus of cell and results in low gene expression. Therefore, a balance between cell introduction and protection for biologic function of shRNA is important (Jiang et al. 2009). In our study, it has been observed that while the free shRNA degraded completely with the effect of DNase I, nanoplexes protected the shRNA well against the degradative effects of DNase I and serum.

In order to test the protection of shRNA by chitosan from serum enzymes such as nucleases and RNases, serum stability assay was performed. As seen in Figs. 2 and 3, free shRNA was rapidly degraded with enzyme and serum but the formation of nanoplexes with chitosan efficiently protected the shRNA from degredation up to 24 h with enzyme and 48 h with serum.




(a)




(b)

Fig. 3: **a:** Serum stability of chitosan/shVEGF nanoplex
1.Standard DNA (λDNA/HindIII), 2.shVEGF, 3.Blank
(chitosan/shVEGF;2/1) 4-10. 0,15,30 min, 1,4,24,48 h
**3b:** Serum stability of free shVEGF
1.Standard DNA (λDNA/HindIII), 2.shVEGF, 3-10. 0,5,15, 30 min,
1,4,24,48 h

### *2.3. Characterization of the nanoplexes*

For nanoplexes different chitosan/shRNA (+/−) ratios were examined using different parameters such as zeta potential and particle size. The cellular uptake properties of nanoplexes can be attributed to the small particle size and excess positive charge that facilitate interaction with cellular membranes (Jiang et al. 2009).

The size of the particles was dependent on the +/- charge ratios. While small particle size (202 nm) was obtained at the lowest charge ratio 0.5/1 (+/−), the use of higher charge ratio (4/1) resulted in relatively larger particle size (469 nm). In our study, the particle size measurements were changed in direct proportion with chitosan concentration added to formulation.

Surface charge of the carrier is also important for the delivery of shRNAs into the cells. Carriers with positive charge are needed due to negative charges of cell membranes. Zeta potential value of free shRNA was $-87.3 \pm 5.3$, it increased to $23.9 \pm 2.9$ mV in the 4/1 (+/−) nanoplex ratio.

By TEM study, chitosan/shRNA nanoplexes were seen globular structure and homogen distribution of nanoplexes. As a result of TEM examination, we observed complex structure which possessed suitable sizes in terms of *in vitro* transfection efficiency (Fig. 4).

### *2.4. In vitro transfection and VEGF inhibition studies*

For *in vitro* transfection study, four cell lines, three of which were cancer cells namely MDA-MB-435, MCF-7, HEK293 and HeLa were tested.

Having considered the results of physicochemical studies performed earlier, nanoplexes showing appropriate properties were selected to compare their transfection efficiencies. Using a fluorescent microscope, cellular transfer ability of nanoplexes were examined with GFP (green fluorescent protein) which was present in plasmid structure. Chitosan/shRNA nanoplexes were prepared in 2/1 ratio and then transferred to the cells. As seen in Fig. 5, with nanoplexes given into the four different cell lines green fluorescence was significantly observed due to GFP expression 48 h after transfection.

Furthermore, to investigate whether chitosan/shRNA nanoplexes were capable of introducing the cell, GFP transfection and distribution were followed by confocal microscopy. The intensity of green fluorescence of the MCF-7 cell line showed the cellular uptake of the nanoplexes within 48 h after transfection (Fig. 6).

In order to test shRNA targeted to human VEGF was specifically silenced VEGF expression. Four different cell lines were used in transfection studies with chitosan/shRNA nanoplexes at the different (+/−) ratios. *In vitro* VEGF concentrations in the cells are given in Fig. 7. The control cells not treated with shVEGF, showed high VEGF concentrations. While VEGF concentration was 412 pg/ml in the MCF-7 untreated control group, it decreased to 166-308 pg/ml after application of

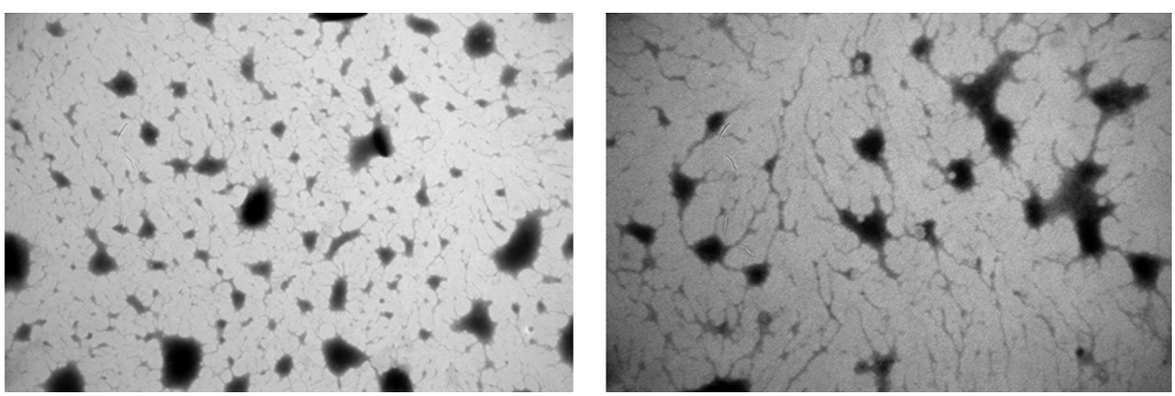


Fig. 4: TEM photographs of the nanoplexes

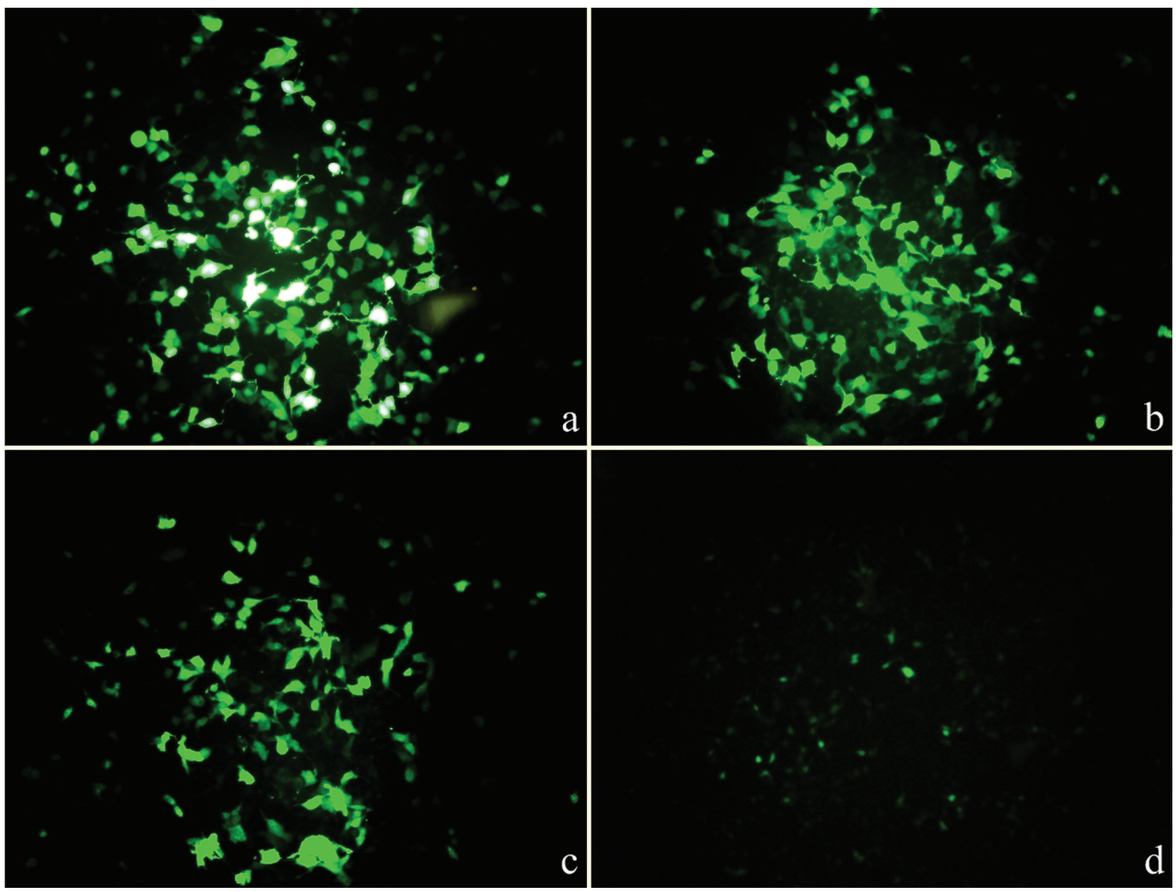



Fig. 5: GFP fluorescence intensity of different cell lines transfected with chitosan/shVEGF (2/1) nanoplexes by fluorescence microscopy. **a**. MCF-7, **b.** HEK293, **c.** HeLa, **d.** MDA-MB435 (x20 magnification)

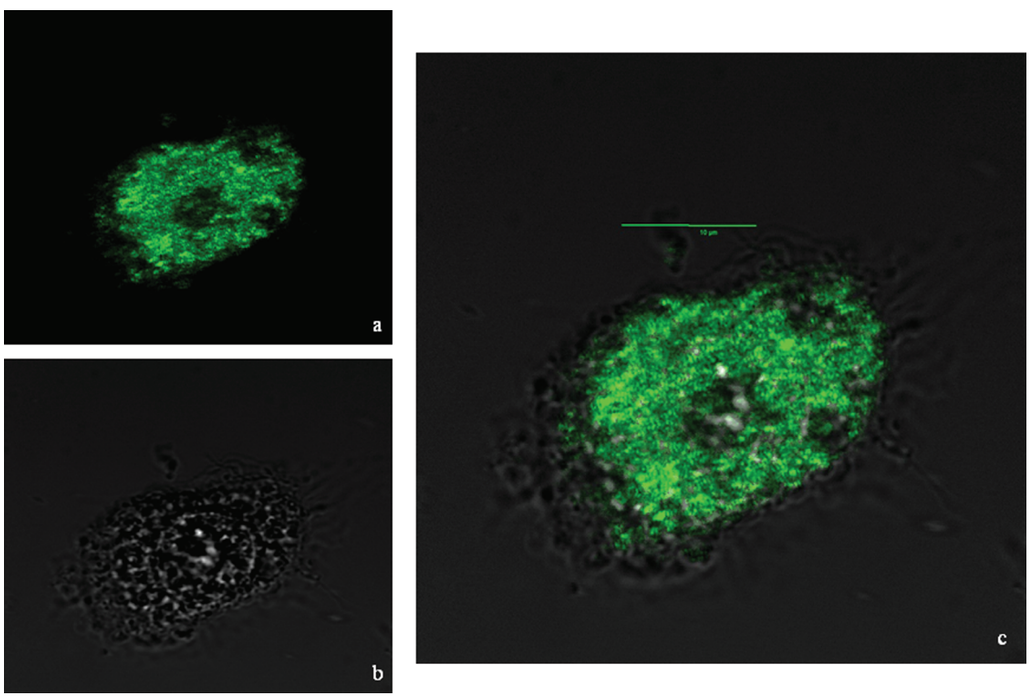



Fig. 6: Cellular localization of chitosan/shVEGF nanoplexes. MCF-7 cells were incubated in the presence of nanoplexes for 48 h. After incubation, samples were examined by confocal laser scanning microscopy. a. green fluorescent protein b. differential interference contrast (DIC) and c. merged image

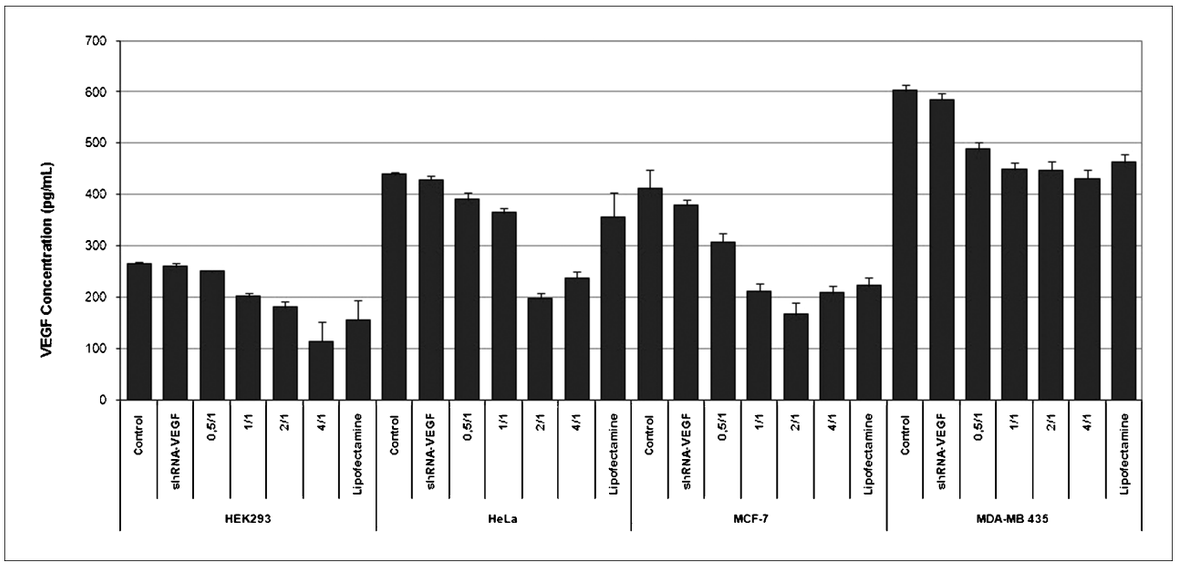



Fig. 7: Inhibition of VEGF production with nanoplexes of chitosan/shVEGF at the different ratios (0,5/1, 1/1, 2/1, 4/1, lipofectamine, free shVEGF) and cell lines (HEK293, HeLa, MCF-7, MDA-MB435). Human VEGF concentration in the conditioned media was determined by ELISA for human VEGF. (As examined gene silencing activity in cells, silencing efficiency was estimated according to its control group for each cell)

chitosan/shVEGF nanoplexes at different ratios. However, free shVEGF application did not affect VEGF protein level in the cells as seen Fig. 7. Except free shVEGF application, VEGF levels in all the cells which transfected chitosan/shVEGF were significantly different from the controls ($p < 0.05$). In comparison between chitosan and lipofectamine as carriers, a higher reducing effect was measured with chitosan/shVEGF in 2/1 ratio than with lipofectamine shRNA complexes in MCF-7 cells (Fig. 7). However, a significant decrease was determined in the VEGF level of cells after chitosan/shRNA nanoplexes application ($p < 0.05$).

Different inhibition levels of VEGF expression were obtained from different cell lines. The highest VEGF suppression was obtained in MCF-7 cells after application of chitosan/shRNA in 2/1 ratio. In the transfection of chitosan/shVEGF at a charge ratio (+/−) of 2/1, *in vitro* silencing effect was MCF-7 (60%) > HEK293 (57%) > HeLa (55%) > MDA-MB435 (29%) in cells transfected by nanoplexes (Fig. 7). While the highest gene knockdown was measured in MCF-7 cell line, the lowest gene knockdown was measured in MDA-MB435 cell line. In conclusion, these nanoplexes showed cell-type dependent transfection efficiency. These differences between cell lines may be due to numbers of VEGF receptors present on the cell surface (Tan et al. 2007). Mao et al. (2001) used HEK293 and HeLa cells and found that the transfection efficiency of chitosan-DNA nanoparticles was cell type dependent. In another study, as reported by Corsi et al. (2003), the cellular membrane composition varied among the different cell types and may facilitate or hinder the binding of complexes and subsequent internalization. This situation has been shown to be important with cationic lipid-DNA complexes (Pouton et al. 1998). The deficiency in proteoglycan synthesis in cells were more difficult to transfect. Thus, knowledge of the composition of the cellular membrane along with receptors distinguished it from the other cells (Corsi et al. 2003). Our results are in accordance with the earlier reports mentioned above (Corsi et al. 2003; Ishii et al. 2001; Mao et al. 2001;Niola et al. 2006).

In conclusion, this study reveals that the use of chitosan, a new delivery system, contributes to the treatment of MCF-7 breast cancer cells by increasing the therapeutic effect of VEGF shRNA. Hence, this study may provide a different approach for breast cancer therapy based on the inhibition of angiogenesis.

## 3. Experimental

### *3.1. Materials*

Chitosan (75 kDa, 75-85% deacetylation) and DNAse I were obtained from Sigma. GenoPure plasmid maxi kit was purchased from Roche. DMEM media and fetal bovine serum used in cell culture were obtained from Biological Industries. The VEGF-A ELISA kit was purchased from Biosource. All materials were used without any additional purification.

### *3.2. Plasmid constructs for short hairpin RNAs*

siRNA-expressing pDNA driven by human 7SK and human CMV-HTLV promoter were constructed from psiRNA-mVEGF vector (InvivoGen, USA). The plasmid psiRNA-mVEGF (shRNA) transcribes a single stranded RNA, which forms stem-loop-structured siRNA, targeted to VEGF mRNA with loop sequences. The target sequence between Cla I and Xba I restriction sites were verified by sequencing. In addition, plasmid construct was included GFP for monitor transfection efficiency to mammalian cells. The plasmid was transformed into an *E. coli* GT116 bacterial strain. The transformed cells were grown in larger quantities of TB broth supplemented with 100 μg/ml Zeocin. The plasmid was purified using a Geno Pure Plasmid Maxi kit. The purity was checked by agarose gel electrophoresis followed by ethidium bromide staining and DNA concentration was measured by UV absorption at 260 nm.

### *3.3. Preparation of Chitosan/shRNA nanoplexes and gel retardation assay*

Chitosan was dissolved in 0.1 M tris acetate/0.1 M acetic acid buffer (pH 5.4) to form a solution of 2.5 mg/ml and 1 mg/ml of shRNA solution was prepared in the TE (Tris-EDTA, pH 8.0) buffer. A series of nanoplexes at various charge ratios prepared by mixing chitosan solutions with shRNA solution. The mixture was vortexed for 15 s and left for 30 min for forming of the nanoplexes completely. The volume of shRNA was constant and the volume of chitosan solution was varied to adjust to theoretical charge ratio (+/−) (molar ratio of amine to phosphate groups) during complex preparation. The nanoplexes were electrophoresed in agarose gel (Salva et al. 2008).

### *3.4. Enzyme and serum stability of nanoplexes*

Protection of complexed shRNA against DNAase I degradation was studied after incubation of nanoplexes with or without of 1 IU DNAse I and DNAse I reaction buffer (10x). Stability of nanoplexes against serum was studied by incubating the samples in 10% fetal bovine serum and 150 mM NaCl solution at 37 °C. Reaction was carried out at 37 °C and aliquots were taken at different intervals. For the inhibition of reaction 0.5 M EDTA was used. The integrity of shRNA was examined using the agarose gel retardation assay. Enzyme and serum stability of free shRNA was also studied at different intervals and analyzed with agarose gel electrophoresis (Koping-Hoggard et al. 2001).

### 3.5. Characterization of the nanoplexes

The diameters and zeta potentials of the nanoplexes were determined using a Malvern Zetasizer (Malvern Instruments 3000 HS, UK). The instrument is equipped with both a particle sizer and a zetameter unit. The samples was measured in PBS in glass cuvettes at 25 °C with a constant angle of 90°. Each measurement was done in triplicate.
Nanoplexes were examined by transmission electron microscopy (TEM, Jeol, Japan) using a technique described in detail (Jiang et al. 2007). Briefly, a small drop (10-20 μl) of sample solution (1 μg/ml pDNA of chitosan nanoplexes prepared in PBS pH 7.4) was deposited on to a copper grid covered by a 0.2% polyvinyl formal (Vinylec K). Excess liquid was blotted away with filter paper. The grids were allowed to dry at room temperature and performed negative staining technique. The grid was stained with 20 μl 0.2% ammonium molibdate solution and left to stand for 5 min at room temperature. All observations were made in zero-loss bright field mode at an accelerating voltage.

### 3.6. Cell culture

MCF-7, MDA-MB-435, HEK 293 and HeLa cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and used for transient transfection experiments. The cells were cultured in DMEM growth medium supplemented with 10% FBS, 100 U/ml penicilin and 100 μg/ml streptomycin at 37 °C in 5% $CO_2$ humidified air. Cells were passed every 3 days on reaching 80% confluency, using a combination of trypsin and EDTA.

### 3.7. In Vitro transfection

Cells were plated on a 6-well plate at a cell density of $1 \times 10^6$ cells/well and incubated overnight. Transfections were performed on cells that were approximately 70% confluent. Prior to transfection, the medium was removed and the cells were rinsed once with phosphate buffered saline (PBS). The wells were refilled with serum-free media. Seven groups were set up: untransfected control group (media without serum and antibiotics), chitosan/shRNA-VEGF nanoplexes in the different ratios groups (0,5/1, 1/1, 2/1, 4/1), free shRNA-VEGF group and lipofectamine group. The cells were transfected as above and incubated for 6 h, after which time, the medium and formulation were removed, the cells rinsed with PBS and the well refilled with serum containing medium. Cells were incubated for an additional 42 h at 37 °C. Cells were harvested at 48 h by removing the medium then adding 250 μl lysis buffer, leaving for 5–10 min by cold-heat treatment (incubation at −20 °C for 30 min in a deep-freezer and then 37 °C for 30 min). Cell debris was removed by centrifugation at 12.000 g. The supernatant was removed.
Lipofectamine 2000 was used according to the manufacturer's procedures. Each well received 5 μg lipofectamine 2000 that was complexed with 2.5 μg of shRNA.
Cell uptake of nanoplexes were investigated using fluorescence (Olympus, BX51, USA) and laser scanning spectral confocal microscope (Leica TCS SP2, Germany) (Murata et al. 2008).

### 3.8. The determination of VEGF amount

ELISA for VEGF was performed in the supernatant of the cell culture suspension, according to manufacturer's instructions (Biosource, California). VEGF protein that accumulated in the culture medium and human VEGF165 protein standard were analyzed using solid phase sandwich ELISA. Absorbance was determined by spectrophotometrically at 450 nm. The inhibition (%) of VEGF secretion by shRNA transfection was calculated from the proportion of VEGF in samples compared with that in controls. All the experiments were repeated thrice and the standard deviation (±) was calculated. To assess the level of knockdown, levels of VEGF in the supernatant from cells transfected with shRNA were compared with levels of VEGF in the supernatant from untransfected cells (Kim et al. 2007).

### 3.9. Statistical analysis

Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student's t-tests.

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