Advanced Pharmaceutical Bulletin, **2012**, 2(1), 7-16
doi: 10.5681/apb.2012.002
http://apb.tbzmed.ac.ir/

Evaluation and optimization of chitosan derivatives-based gene delivery system via kidney epithelial cells

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ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 6 Jan 2012
Accepted: 26 Jan 2012
ePublished: 15 Feb 2012

Keywords:
Chitosan derivatives
Gene delivery
Epithelial cells

ABSTRACT

***Purpose*:** Non-viral vectors have been widely proposed as safer alternatives to viral vectors, and cationic polymers have gained increasing attention because they can form self-assembly with DNA. Chitosan is also considered to be a good candidate for gene delivery systems, since it is already known as a biocompatible, biodegradable, and low toxic material with high cationic potential. However, low solubility and transfection efficiency need to be overcome prior to clinical trial. In this work, we focus on alkyl modified chitosan which might be useful in DNA condensing and efficient gene delivery. ***Methods*:** N, N- Diethyl N- Methyl (DEMC) and N- Triethyl Chitosan (TEC) were synthesized from chitosan polymer. In order to optimize the polymers for gene delivery, we used FITC-dextran (FD). Then the optimized polymer concentrations were used for gene delivery. Fluorescent microscope was used, in order to evaluate the polymers' efficiency for gene delivery to human embryonic kidney epithelial cells (HEK 293T). ***Results*:** This modification increased chitosan's positive charge, thus these chitosan derivatives spontaneously formed complexes with FD, green fluorescence protein plasmid DNA (pEGFP), red fluorescence protein plasmid DNA (pJred) and fluorescent labeled miRNA .Results gained from fluorescent microscope showed that TEC and DEMC were able to transfer FD, DNA and miRNA (micro RNA) to HEK cell line. ***Conclusion*:** We conclude that these chitosan derivatives present suitable characteristics to be used as non-viral gene delivery vectors to epithelial cells.

Introduction

In the past decade, Cationic polymers have been proposed as an alternative approach to the viral vectors. Generally, cationic polymers form efficient complexes with DNA and interact with cells. Polymer/DNA complexes are more stable than cationic lipids, and they protect DNA against nuclease degradation. [1] Examples include diethylaminoethyl dextran [2], poly(L-lysine) (PLL) [3], polyethylenimine (PEI) [4], gelatin [5], polyamidoamine dendrimers [6], and chitosan. [7] Both PEI and the dendrimers are effective gene carriers, but both are synthetic and not biodegradable, which means that their potential toxicity is a concern. Although biodegradable, PLL forms polyplexes with lower transfection efficiency than that of PEI and the dendrimers.

Among non-viral vectors, chitosan has been considered to be a good gene carrier candidate, since it is known as a biocompatible, biodegradable, and low toxic material with high cationic potential [8], and it has functional groups that allow simple coupling of extracellular and intracellular targeting ligands. [9] However, the low specificity and low transfection efficiency of chitosan must be overcome for its use in clinical trials. Up to now many chemical modifications have been done on chitosan. These chemical modifications include hydrophilic [10], hydrophobic [11], pH-sensitive [12], thermosensitive [13] and cell-specific ligand [14] groups for enhancement of cell specificity and transfection efficiency of chitosan in vitro. One of the subtypes of hydrophobic modification of chitosan is alkylated chitosan(ACSs).

Alkylated chitosans synthesized for gene deliveries are, N-dodecylated chitosan (NDC) [15], alkyl bromide [16] and trimethlyated chitosan oligomers [17]. Hydrophobic units in the polymeric carriers may assist dissociation of polymer/DNA complexes, to facilitate release of DNA which otherwise would be strongly bound through ionic interactions between cationic units and phosphates of DNA. [18] These favorable characteristics of the hydrophobic units lead to higher transfection

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efficiency of chitosan than polymer systems using only ionic interactions. In the hydrophilic modification of chitosan by alkylation, Kean et al. proposed that TMC, a quaternized form of the chitosan, makes the chitosan soluble over a wide pH range, increases gene –polymer intraction and increases its transfection efficiency with less toxicity. [19]

Human Embryonic Kidney Epithelial Cells (HEK 293 Line), are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. An important variant of this cell line is the 293T cell line that contains, in addition, the SV40 Large T-antigen, that allows for episomal replication of transfected plasmids containing the SV40 origin of replication. This allows for amplification of transfected plasmids and extended temporal expression of the desired gene products. [20]

The aim of the present work is to show the effect of another chemical modification of chitosan in gene delivery. DEMC and TEC were synthesized from chitosan polymer. In order to optimize the polymers for gene delivery, we used FITC-dextran (FD). Then the optimized polymer concentrations were used for gene delivery. Fluorescent microscope was used, in order to evaluate the polymers' efficiency for gene delivery to human embryonic kidney epithelial cells (HEK 293T).

Materials and Methods

Materials

Low molecular weight chitosan from Primex, Iceland. Ethyliodide, methyl iodide, and sodium borohydride were obtained from Sigma (Vienna, Austria). Sodium hydroxide, *N*-methyl pyrrolidone (NMP) and sodium iodide were purchased from Merck (Darmstadt, Germany). HEK 293T cell line was purchased from NCBI (Tehran, Iran). Flourescein isothiocyanate-dextran (sigma, USA)**,** Plasmid extraction kit (Fermentas Miniprep Kit, Lithuania), RPMI 1640 (Gibco-BRL, UK), FBS (Fetal Bovine Serum, Gibco® In vitrogen Ltd, UK), Lipofectamine™ 2000 (in vitrogen, USA), Scramble-miRNA (Exiquon, USA), DMEM (Dulbecco's modified Eagle, Gibco-BRL, UK), all were obtained from stem cell technology institute (Tehran, Iran).

Synthesis and characterization of N-triethyl chitosan

TEC was prepared by a method reported by Avadi et al [21] At the beginning, we distributed chitosan. (DD=97%) in *N*-methyl pyrrolidone and it was mixed at room temperature. Then sodium hydroxide, sodium iodide and ethyl iodide was added to the mixture and it was heated at 60 °C for 6 hours under stirring. The product chitosan–N+(CH2CH3)3I– was precipitated with acetone and separated by centrifugation. In order to separate the N-methyl pyrrolidone between the TEC chains and get a better NMR, the precipitate was left for one week in acetone under gentle stirring. To exchange I– with Cl–, the polymer was dissolved in 10% aqueous sodium chloride solution and stirred for 3 h. The polymer was precipitated with acetone, centrifuged and dried to obtain a white water-soluble powder. The polymer structure, the degree of quaternization and zeta potential were characterized by H-NMR and Malvern zeta sizer.

Preparation and characterization of N-diethylmethyl chitosan

DEMC was prepared by a two-step method reported by Avadi et al. [22] In the first step chitosan was dissolved into 1% acetic acid solution and formaldehyde solution was added. After stirring for 1 h, NaBH4 was added and the stirring continued for 16 h. Then the solution pH was adjusted to 10 by adding 1 M NaOH solution and a white precipitant was yield. In the second step, methyl chitosan was dispersed in *N*-methyl pyrrolidone and after 4h of sttiring, sodium hydroxide; ethyl iodide and sodium iodide were added. Reaction was carried out with stirring for 6 h at 60 C. Finally acetone was added and the precipitant of chitosan derivative was collected. In order to separate the *N*-methyl pyrrolidone between the TEC chains and get a better NMR, the precipitate was left for one week in acetone under gentle stirring. For exchanging I- with Cl-, the polymer was dissolved in sodium chloride (10%) solution. The polymer was precipitated with acetone, centrifuged and dried to obtain a white water-soluble powder. The polymer structure, the degree of quaternization and zeta potential were characterized by H-NMR and Malvern zeta sizer.

Plasmid preparation

The EGFP and Jred plasmids were used to monitor gene transfer and transgene expression after transfection. The plasmid was transformed in Escherichia coli ($DH_{5\alpha}$). After thawing DH5α on ice for 15 minutes, it was aliquot (100 uL) into pre-chilled 1.5 mL microfuge tubes. 10 ng/uL of plasmid was added to 100 uL cells and mixed. DH5α / plasmid mix were incubated on ice for 20 minutes and heat shocked at 45^{o}C for 42 seconds. 800 ul of LB (liquid broth) was added to the cells with shaking for 1h, and then centrifuged. Transformed cultures were plated on LB plates containing 100 µg/ml ampicillin and incubated for 12-16 h. 5 ul of the cells were mixed with 5 ml LB containing ampicillin and gently mixed at 37^{o}C. After 16 h the transformed bacteria were centrifuged and the plasmid was isolated via Plasmid Miniprep Kit. The Plasmid concentration and purity were determined using BioPhotometer Eppendorf (Hamburg, Germany) and electrophoresis on 1.5% agarose gel. The gels were stained with ethidium bromide and photographed on a UV transilluminator (Uvidoc, Bridgeville, UK). The plasmid size and purity are showen in figure 1. Compared to 1kb DNA Ladder (GeneRuler™, Fermentase) the plasmid is pure and its size is approximately 7 kbp. The plasmid concentration was 1 µg/µl.

HEK293T cell line preparation

HEK293T cells were cultured in plates. The cells medium was Dulbecco's modified Eagle (DMEM) with 10% (V/V) fetal bovine serum (FBS), 100 u/ml penicillin and 100 u/ml streptomycine. They were cultured at 37C in a humidified atmosphere of 5% CO2 and 95% air. The cells were used for transfection at 70-80% confluency, 24 h post-seeding.

Fluorescein isothiocyanate-dextran transfection

In order to optimize the system for gene delivery, we used Flourescein isothiocyanate-dextran **(**FD) (Figure 2). HEK 293T cell line, which are the standard cells for transfection, were used for system optimization. In order to calculate the amount of TEC and DEMC for transfection we used different polymer/FD ratios. To obtain a complex between FD (20 ug/ml) and each quaternized derivative, we prepared different polymer concentrations. The polymer concentration (polymer/FD (w/w) ratio) that were used for TEC and DEMC on HEK cell line were: 0.0001% (0.05), 0.0004% (0.2), 0.002% (0.8), 0.003% (1.6), 0.006% (3.2), 0.01% (6.4), 0.03% (12.8), 0.05% (25.60), 0.1% (51.20), 0.25% (125), 0.5% (250) and 1% (500) and 2% (1000). Polymer/FD complexes were prepared by adding the polymer solution to FD solution (1:1) and incubating for 2h at room temperature under gentle stirring on incubator stirrer.

600000 cells/cm^2 HEK cells were cultured in 6 well plates as described above. Prior to transfection, culture medium was removed and culture medium without FBS was added. After 2h polymer/FD complexes were added to the cells. Then the cells were incubated at 37^{o}C on low speed shaker for 15 min. The cells were seen with fluorescent microscope after 5h.

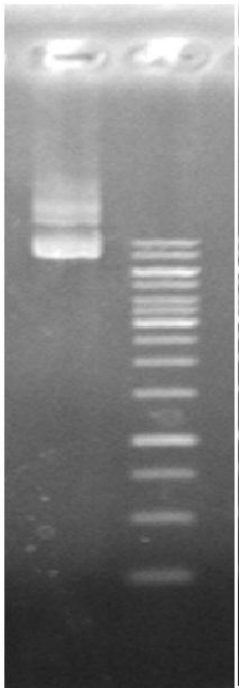

Figure 1. Plasmid size and purity compared to DNA ladder.

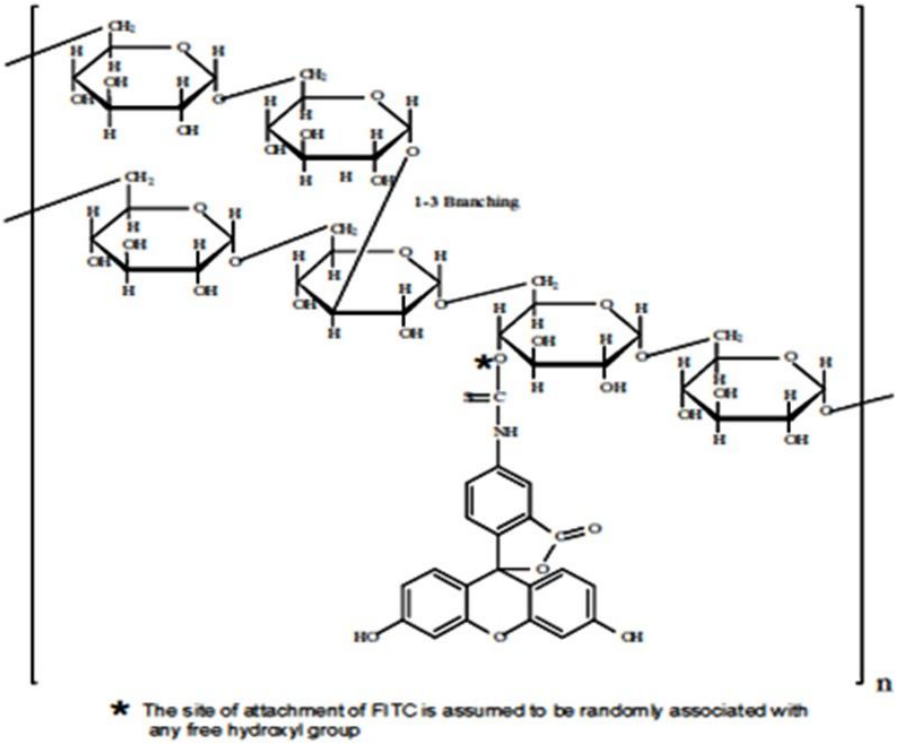

Figure 2. FITC-dextran structure

miRNA transfection

The miRNA was brought from Exiquon Company (Control, miRCURY knockdown probe, 5 nmol in 200 µL, 5`-fluorescein labeled) (Figure 3). The chitosan derivatives (TEC and DEMC) were dissolved separately in DMEM without serum. Polymer/miRNA complexes were prepared by adding polymer solution to miRNA solution (50 nM) at equal volume and quickly mixed before incubating them at room temperature for 30 min.

2.5×10^4 HEK293T cells were cultured in 96 well plates. Prior to transfection, culture medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). 100 µl/well of polymer–miRNA complexes, miRNA alone or Lipofectamine 2000–miRNA (prepared according to protocol) complexes were added to wells. The cells were incubated at 37C. The transfected cells were detected with fluorescence microscope after 5h.

GTGTAACACGTCTATACGCCCA

5`-fluorescein labeled

Figure 3. Scramble-miRNA

Plasmid (pEGFP and pLEX-JRed) transfection

Two kinds of plasmids (1 ug) were used for HEK cell transfection with TEC and DEMC: 1) pLEX-JRED

(from stem cell technology institute (Tehran, Iran)) which produced red fluorescent protein and 2) pEGFP which produced green fluorescent protein after transfection. Thus these plasmids could easily be detected and used for transfection optimization. (Figure 4, 5)

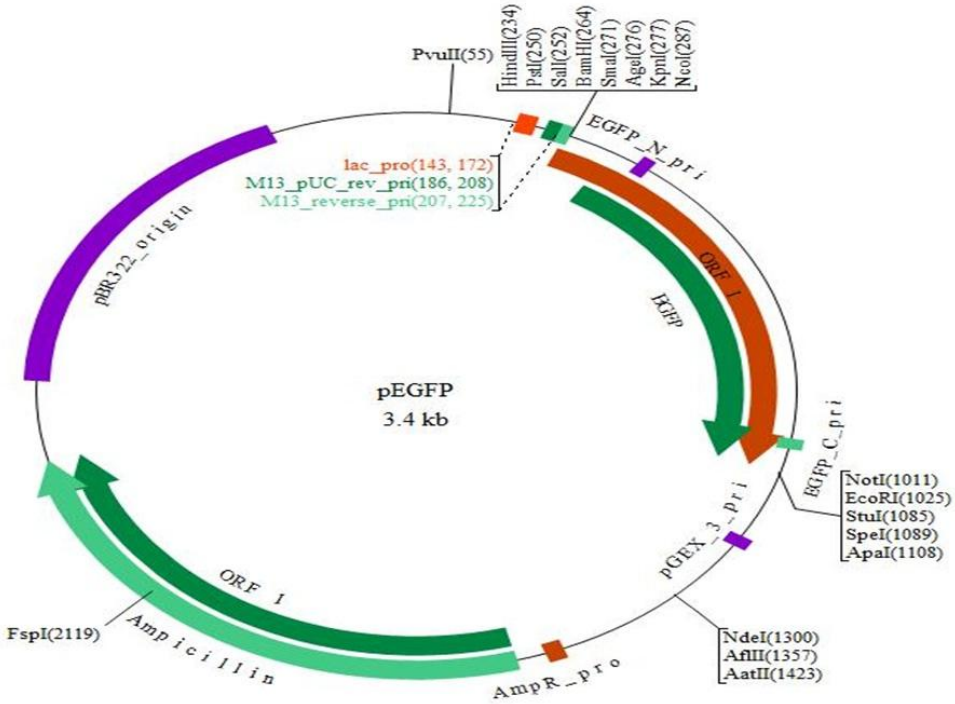

Figure 4. pEGFP (Enhanced green fluorescent protein plasmid)

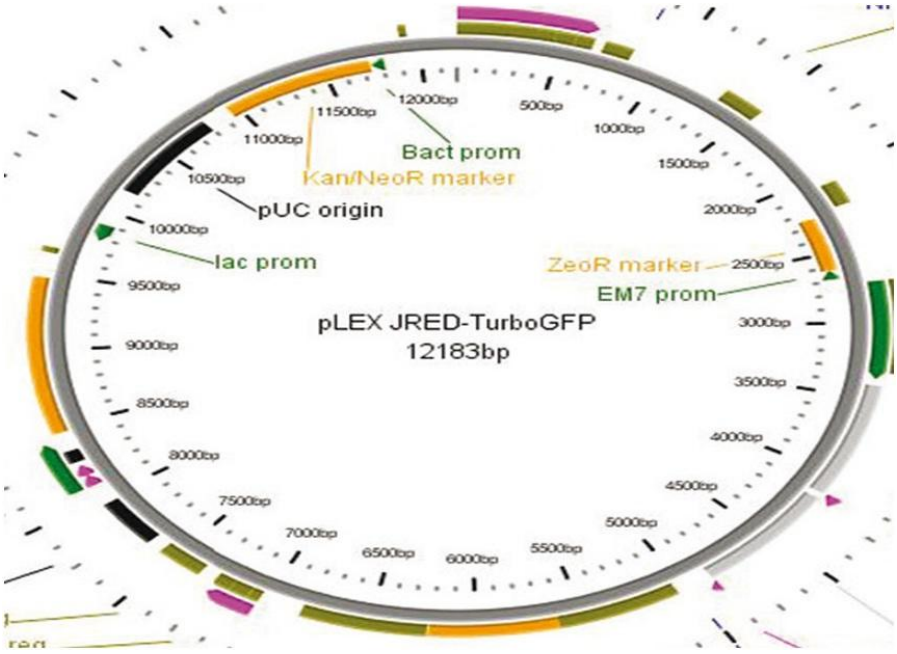

Figure 5. Jred (Red fluorescent protein plasmid)

HEK293T cells were cultured in 6 well plates. Prior to transfection, culture medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with plasmid alone, polymer/plasmid complexes with both chitosan derivatives and also Lipofectamine 2000-plasmid (prepared according to protocol) at 37^{o}C. After 5 h the formulations were removed, the cells were rinsed with PBS and grown in culture medium for 24 h to allow for GFP expression and detected with fluorescent microscope.

Results and discussion

Polymer preparation

The H-NMR spectrum, data and polymer peaks for TEC and DEMC can be seen in the below figures. (Figure 6, 7)

For TEC, the triple signal at 1.2 ppm was attributed to the CH3 groups of the ethyl substituents, while the CH2 groups at the quaternized site are superimposed by the 2-H and 6-H protons of the polysaccharide backbone. The intense band at 4.8 ppm was due to HDO (solvent). The integral of CH3 of ethyl groups versus the other protons was used to calculate the degree of quaternization. According to the NMR peak, the percent of quaternization of TEC is 21%. Also TEC's zeta potential is +36.5 mV.

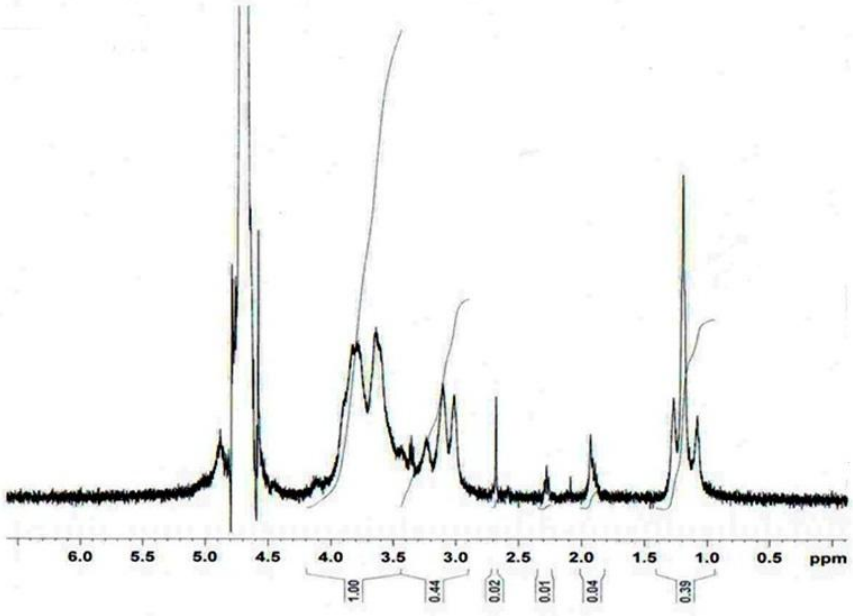

Figure 6. H-NMR of TEC

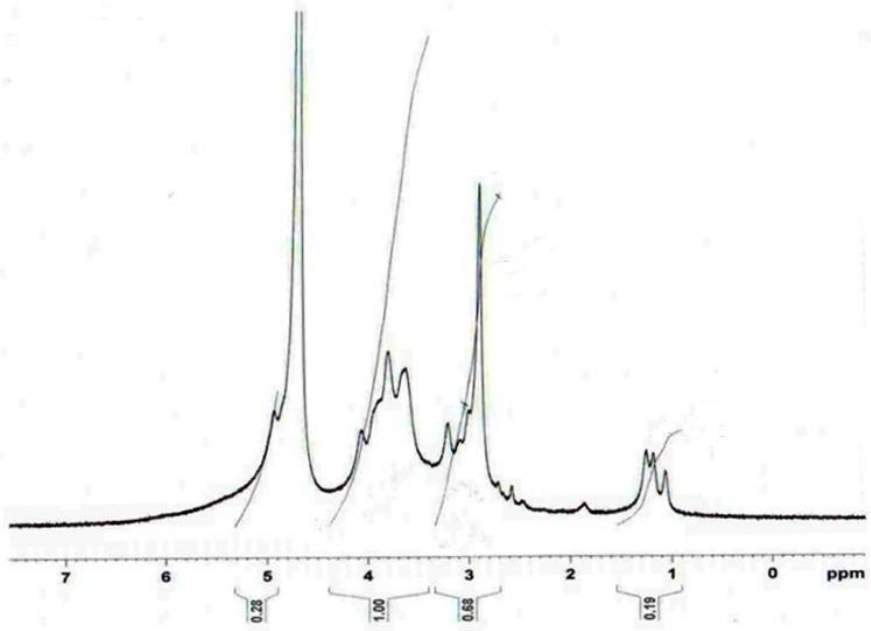

Figure 7. H-NMR of DEMC

According to DEMC H-NMR, the signal at 1.3 ppm was attributed to CH3 groups of the ethyl substituent and the signal at 3 ppm is related to the CH3 group of methyl. While H2-H6 protons of the polysaccharide backbone are superimposed by the CH2 groups. The intense band at 4.8 ppm was related to HDO. The integral of CH3 of ethyl groups versus the other protons was used to calculate the degree of

quaternization. According to the NMR peak, the percent of quaternization of DEMC is 21%. Its zeta potential is +58 mV.
According to results, because of their positive charge, both polymers are suitable for complex formation with negative charged Flourescein isothiocyanate-dextran and plasmids.

Cell transfection with FD/chitosan derivatives complexes

After research it was found that the best FD transfection with TEC and DEMC is at 2% (polymer/FD: 1000) polymer concentration. Transfection was completed after 5h. The transfected HEK cells with TEC/FD and DEMC/FD were seen with fluorescent microscope (Figure 8-10). As it can be seen in these figures the cells transfected with FD are green fluorescent.
FITC-dextran (FD) is primarily used for studying permeability and transport in cells and tissues. The advantages of FD are that just like nucleic acids. It has negative charge and because of its fluorescence part, it can easily be detected inside the cells. Also in a study done by Sushma et al. preparation of thiol-modified gelatin nanoparticles for intracellular DNA delivery was evaluated. [23] In this study the gelatin concentration used for gene delivery was 1% (w/v). Compared to our study, which 2% (w/v) of DEMC and TEC was used for FD transfection, lower concentration of gelatin is needed for FD transfection. Up to now no studies have been done on FD transfection with chitosan derivatives.

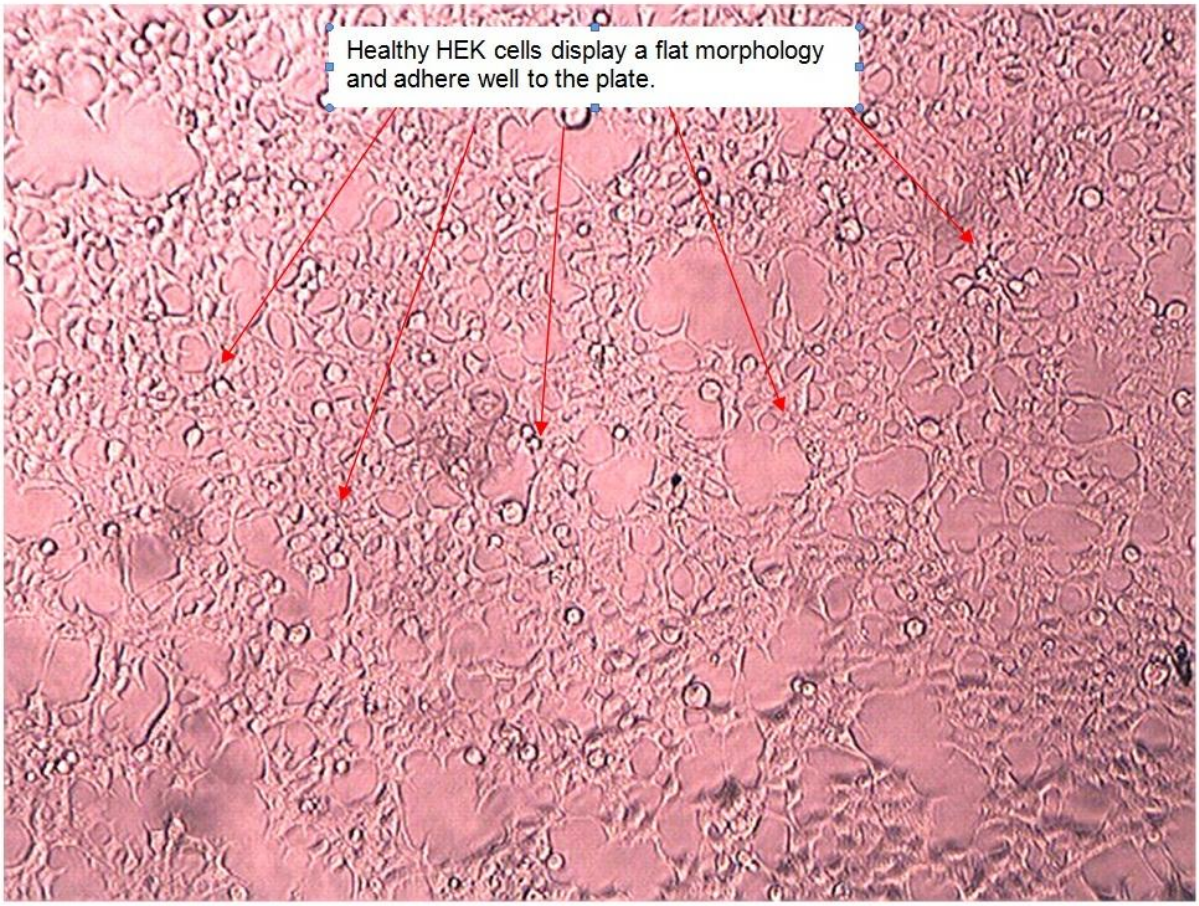

Figure 8. HEK 293T cells (Human Embryonic Kidney Epithelial Cells)

miRNA transfection

Since the miRNA was fluorescein labeled so, like fluorescence dextran (FD), it was detected after 5 hours in the cell cytoplasm. As it can be seen in the figures below (Figure 11, 12), the miRNA can be seen in the cells as green fluorescent dots. The transfected cells with lipofectamine are seen in figure 13. As it can be seen there was no obvious transfection for uncomplexed miRNA (Figure 14).
The positively charged head group of polymers makes electrostatic complexes with the negatively charged phosphate ions on the base backbone on miRNA. Thus, DEMC/miRNA and TEC/miRNA complexes are prepared by this mechanism. From the gained results, it is concluded that TEC and DEMC could be used for miRNA delivery to epithelial cell lines. In a study done by Katas et al. chitosan nanoparticles for siRNA delivery was developed by chitosan hydrochloride and chitosan glutamate and it was concluded that low concentration and low molecular weight of chitosan is needed for RNA delivery. [24]

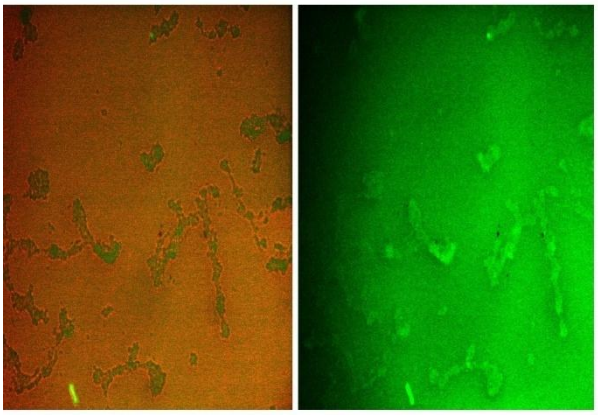

Figure 9. HEK 293T cell line transfected with TEC/ FD complexes.

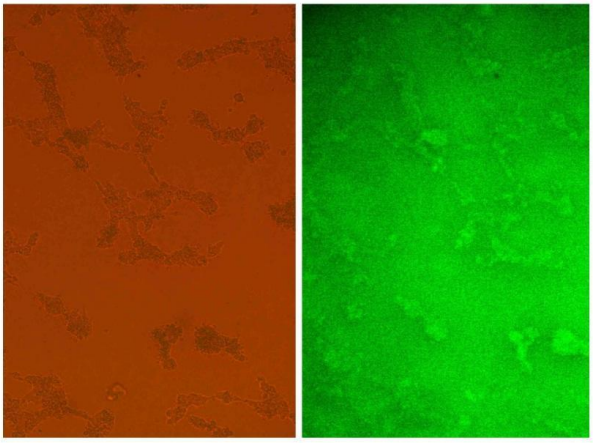

Figure 10. HEK 293T cell line transfected with DEMC/ FD complexes.

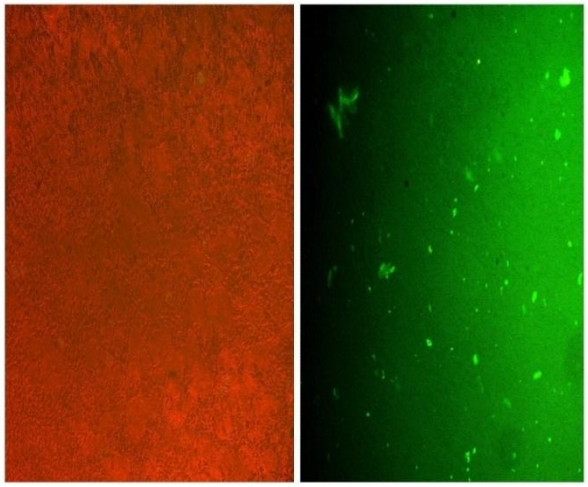

Figure11. HEK 293T cell line transfected with TEC/ miRNA complexes.

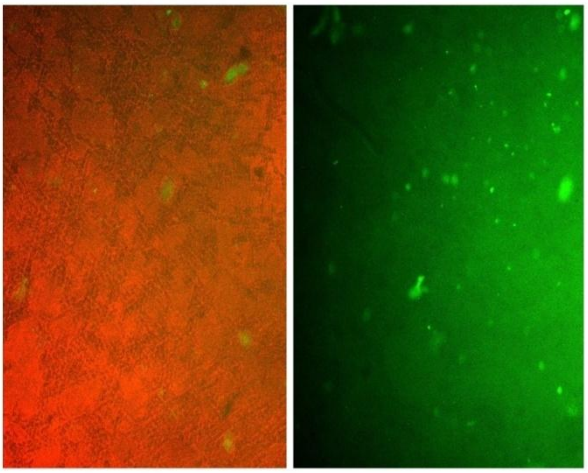

Figure 12. HEK 293T cell line transfected with DEMC/ miRNA complexes.

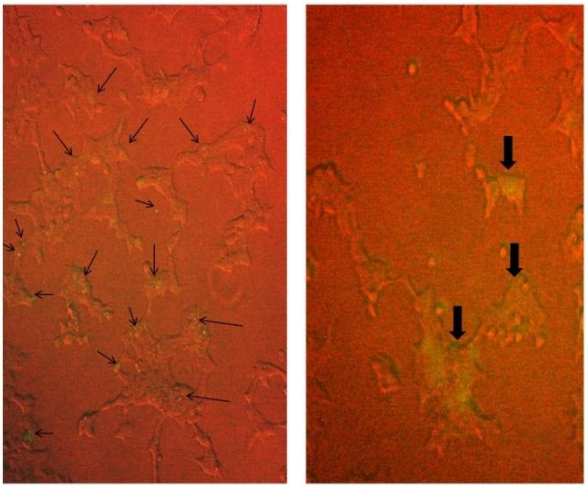

Figure 13. HEK 293T cell line transfected with lipofectamine/miRNA

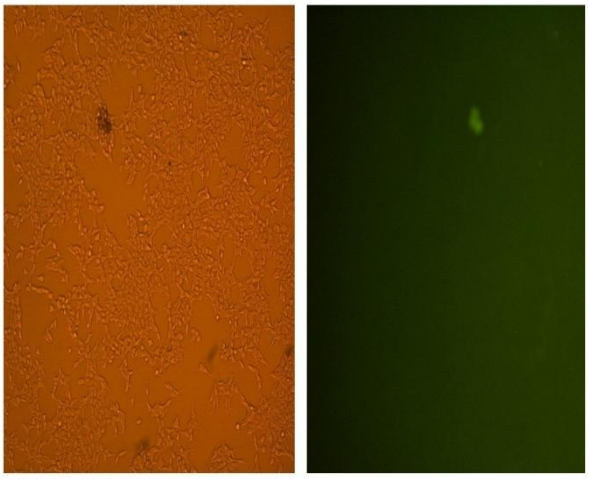

Figure 14. HEK 293T cell line transfected with miRNA

Plasmid transfection

As it can be seen in Figure 15-19, approximately 80-90% of the HEK cells are transfected with TEC and DEMC complexed with pEGFP and pJred. Compared with lipofectamine (positive control) and plasmid transfected (negative control), the results with pEGFP and pJred are seen as green flouresent protein and red flourecent protein.

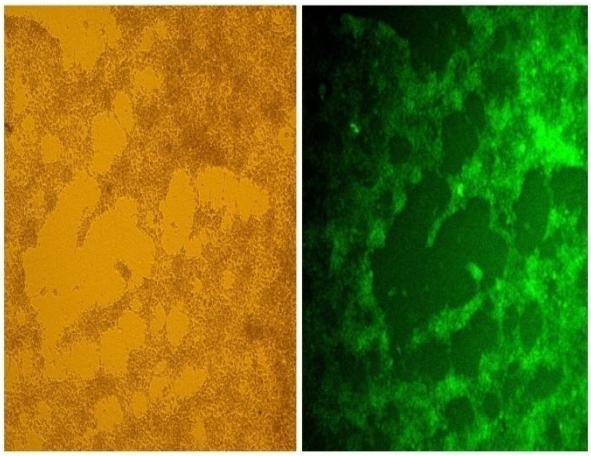

Figure 15. HEK 293T cell line transfected with TEC/pEGFP

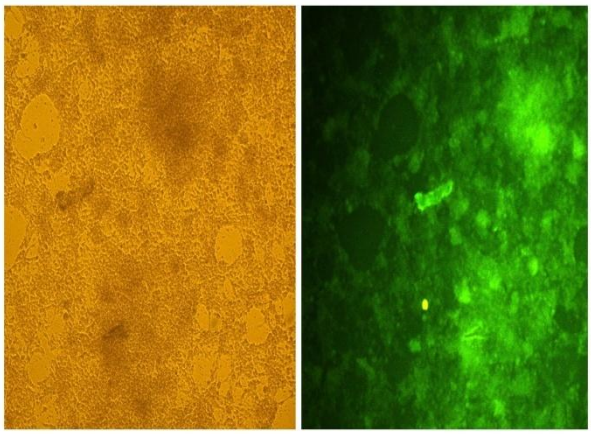

Figure 16. HEK 293T cell line transfected with DEMC/pEGFP

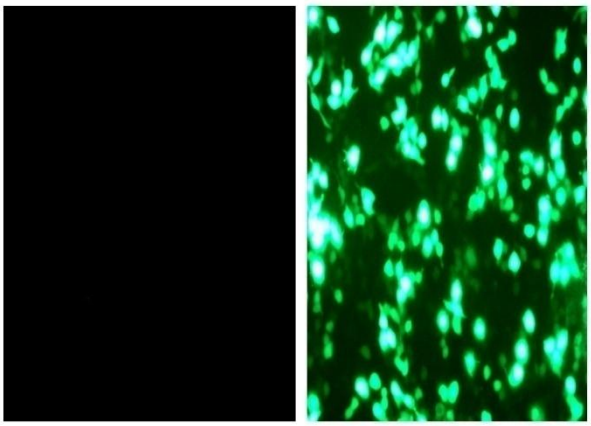

Figure 17. HEK 293T cell line transfected with lipofectamine/pEGFP (right) and pEGFP (left)

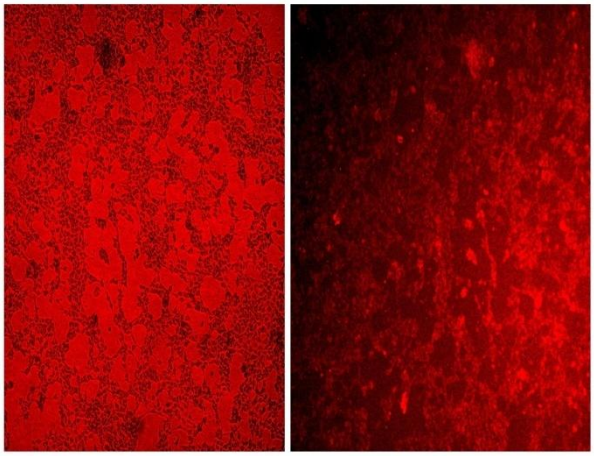

Figure 18. HEK 293T cell line transfected with TEC/Jred

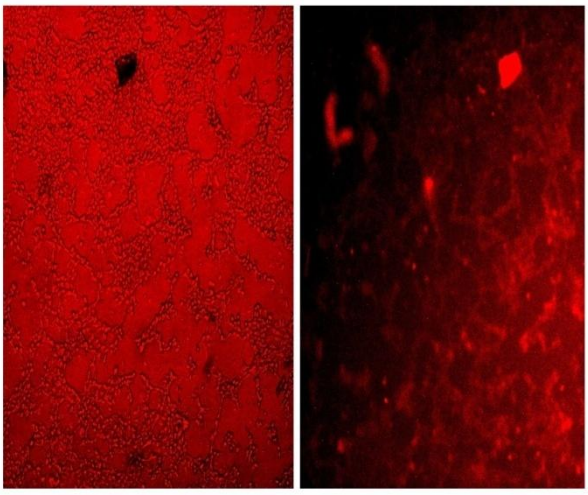

Figure 19. HEK 293T cell line transfected with DEMC/Jred

As stated in the introduction many studies have been done on chitosan derivatives for gene delivery. Among these studies Kiang et al. formulated chitosan-DNA nanoparticles with poly(propyl acrylic acid) which enhanced gene expression. [25] Also Mao et al. increased transfection to HEK cells by attaching transferrin to chitosan. [26]

So up to now, according to the transfection results, the polymer concentration required for cell transfection is 2%. With this polymer/concentration we had transfection in most of the HEK cells. Triethyl chitosan (TEC) and Diethylmethyl chitosan (DEMC) are quaternized and more hydrophobic then TMC so we could use the advantages of both hydrophobic and hydrophilic modification of chitosan in gene delivery. Quaternizing the polymer increases gene –polymer intraction and increases its transfection efficiency and hydrophobic modifications of chitosan increases transfection efficiency by modulating complex interactions with cells, such as adsorption on cell surfaces and cell uptake.

Conclusion

Based on the assumption that quaternization may increase the DNA condensing ability of chitosan, we have prepared quaternized derivatives of chitosan polymer, Triethyl chitosan (TEC) and Diethylmethyl chitosan (DEMC) .These derivatives proved to transfect HEK 293T cells to a high extent. Considering that HEK cells are the standard cell line for transfection evaluation the transfection results in this cell line could be used in order to transfect other epithelial cell lines including pancreatic cancer cells (the results will be published). Up to now these results indicate that these partially quaternized chitosan derivatives are promising agents to be used in gene and miRNA, which are used widely in cancer therapy [27,28], delivery.

Acknowledgements

The authors would like to acknowledge the stem cell technology institute (Tehran, Iran) for generous use of equipment.

Conflict of interest

The authors report no conflicts of interest.

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