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Chitosan nanoparticles as a potential nonviral gene delivery for HPV-16 E7 into mammalian cells

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Abstract

Chitosan nanoparticles (CS NPs) were prepared as a carrier for Human papillomavirus type 16 HPV-16) E7 gene and their gene transfection ability were evaluated *in vitro*. The plasmid expressing green fluorescent protein (pEGFP) was used as a reporter gene. Gel electrophoresis demonstrated full binding of CS NPs with the pDNA. The transfection of CS-pEGFP NPs was efficient in CHO cells and the expression of green fluorescent proteins was well observed. The expression of E7 proteins was confirmed under SDS-PAGE and western blot analysis. As a conclusion CS NPs may serve as an effective nonviral carrier for delivery of nucleotides into eukaryotic cells.

Keywords: chitosan NPs, CHO cell line, gene delivery, HPV-16 E7

Introduction

Human papillomavirus type 16 (HPV-16) infections in humans are associated with most cervical cancers, and expression of the early oncogenic proteins E6 and E7 is required to maintain the transformed state of the tumor cell. E6 and E7 proteins are co-expressed in most HPV-containing cervical cancers. These oncoproteins are believed to sustain the malignant state by disrupting the cell cycle regulatory proteins p53 and Retinoblastoma protein, pRb. Specifically, HPV-16 E7 binds and destabilizes pRb, allowing unscheduled entry into the S-phase of the cell cycle (zur Hausen 1996). Therefore, E7 is an attractive target not only for development of cancer vaccine to prevent cervical tumors, but it is also a good model for exploration of immunotherapeutic approaches to stimulate immune responses against existing HPV-16-associated malignancies (Bosch et al. 1995, Melief et al. 2000). Specific immunity against HPV-16-transformed tumors in murine models has been achieved by a number of vaccine strategies such as gene therapy with E7 (Duggan et al. 1998, Chen et al. 2000).

Gene therapy, the transfer of genes for therapeutic benefit, offers the potential for permanent cure of cancers. The success in gene therapy depends on an efficient strategy for the delivery of nucleic acid into the target cells. Both viral and nonviral vectors have been used in clinical trials to treat several forms of cancers (Somia and Verma 2000). Although, nonviral vectors are less efficient in successfully delivering the genes into target cell, they enjoy the advantage of higher biodegradability and lower toxicity. Nonviral carriers are less immunogenic and more convenient (Pouton and Seymour 2001, Ferber 2001).

Positively charged polymers (referred to as polycations) are promising carriers among nonviral vectors. Many cationic polymers, such as cationic polysaccharides, cationic liposomes, dendrimers, polylysine, polyethyleneimine, and poly (a-(4-aminobutyl)-L-glycolic acid), have been studied for gene delivery. Amongst them, chitosan (CS) is a particularly attractive cationic vector derived from polysaccharides. Polysaccharides are the most abundant polymers in nature and CS exhibits desirable characteristics such as biocompatibility and biodegradability that is essential for gene delivery (Bozkir and Saka 2004). CS is also nonimmunogenic and nontoxic. The positive charge of CS allows for easily prepared NPs through electrostatic interaction between negatively charged pDNA and positively charged CS. CS has extensively been studied for drug delivery, therefore its advantageous characteristics for delivery both *in vivo* and *in vivo* have been broadly investigated.

CS is a linear cationic polysaccharide composed of β1 4 linked glucosamine and partially containing N-acetylglucosamine as shown in Figure 1 (Onishi and Machida 1999, Wu et al. 2005).
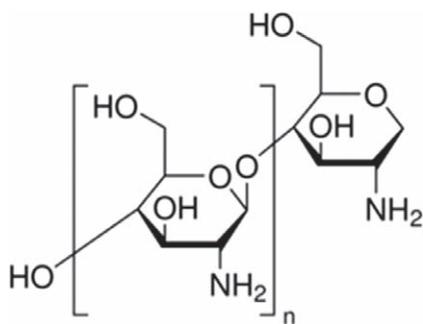
Figure 1. Chemical structure of chitosan.

In this study chitosan nanoparticles (CS NPs) were acquired by the process of ionotropic gelation based on the interaction between the negative groups of the pentasodium tripolyphosphate (TPP) and the positively charged amino groups of CS (Vila et al. 2004). The particles are hydrophilic, biocompatible, and biodegradable. These NPs are ideal for therapeutic targeting (Agnihotri et al. 2004). Furthermore, they bear a high density of positive charges along its polymeric chain, which is necessary to condense the negatively charged DNA into compact structures, giving them protection during cellular trafficking and promoting its uptake to cells (Rudzinski and Aminabhavib 2010).

In this study, a CS vector with improved transfection efficiency *in vitro* cell culture system as well as *in vivo* mouse model system was prepared. The transfection efficiency was investigated by examination of EGFP expression in CHO cells treated with CS–pEGFP NPs through fluorescence microscopy. Cell viability was also evaluated in CHO cells. Based on our findings, CS–HPV-16 E7 NPs may be a promising carrier for gene therapy in cancer.

Materials and methods

Plasmid propagation and extraction
Bacterial strain *Escherichia coli* DH5α (Pasteur Institute of Iran) was used for propagation and preparation of the pEGFP-N1 (Clontech Laboratories) and pcDNA3.1 (Invitrogen, Carlsbad, CA) plasmids. pEGFP-N1 encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (Ghaemi et al. 2010). For constructing pcDNA3.1 HPV-16 E7 expression vector, the HPV-16 E7 gene was digested with EcoRI and XhoI from the pUC18 vector using suitable restriction enzymes to be ligated into pcDNA3.1 backbone, as we described before (Ghaemi et al. 2010). The plasmids were extracted according to the manufacturer's instructions and dissolved in endotoxin-free Tris-EDTA (Sigma, St. Louis, MO).

CS depolymerization
CS with the deacetylation degree of 95% and the molecular weight (MW) of 360 kDa was purchased from the Primex (Karmoy, Norway) and depolymerized by a chemical reaction as previously described (Akhlaghi et al. 2010) to obtain the low molecular weights of CS. Briefly, 10 ml of NaNO2 solutions (8 mg/ml concentrations in deionized water) was added to the CS solution (2 g in 100 ml of acetic acid 6%

vol/vol) at room temperature under magnetic stirring for 1 hour. Low molecular weight CS was precipitated by raising the pH to 9 by addition of 4 M NaOH drop wise. The white-yellowish solid was filtered and washed thrice with acetone (Merck) and dissolved in a minimum volume of acetic acid 0.1 N (Merck). Purification was performed by subsequent dialyses (Sigma dialyses tubes MW cutoff 12 kDa) against 1 L deionized water (twice for 90 minutes and once overnight). The low molecular weight CS was freeze-dried and then stored at 4°C.

Molecular weight measurement
The molecular weight of depolymerized CS was determined by gel permeation chromatography (GPC) using pullulane standards (Atyabi et al. 2008). All chromatograms were generated on an Agilent 1100 Liquid Chromatographer (Agilent Technologies) and the eluting fraction was followed up by a refractive index signal detector. The mobile phase comprised of 0.2 M acetic acid and 0.1 M sodium acetate (both from Merck) at a flow rate of 4 ml/min. The chromatographic procedures were performed at 23°C. Thirty milligrams of depolymerized CS was dissolved in 10 ml of 0.2 M acetic acid and 0.1 M sodium acetate. The mixture was analyzed by the GPC system using the narrow method.

CS NP preparation
CS NPs were prepared according to the ionotropic gelation technique with TPP cross-linker (Merck, Darmstadt, Germany) as described previously (Yousefpour et al. 2011). CS and TPP were separately dissolved in ultrapure water in order to obtain CS/TPP ratios 3/1. CS NPs were immediately produced by adding a fixed volume of TPP (1 ml) solution drop wise to a fixed volume of CS solution (3ml) under 1000 rpm magnetic stirring at room temperature.

Size and zeta potential determination
The hydrodynamic mean diameter of prepared CS NPs was determined by dynamic light scattering using Nano-Zetasizer (Malvern Instruments, Worcestershire, United Kingdom) with a wavelength of 633 nm at 25°C with an angle detection of 90°. The samples were diluted with freshly filtered deionized water and all measurement was done thrice.

The zeta potential of prepared CS NPs was determined using laser Doppler electrophoresis using Zetasizer (Nano-ZS Malvern Instruments). All samples were diluted (1:5) in deionized water and each sample was measured thrice.

Scanning electron microscopy
The surface morphology of prepared CS NPs was observed using scanning electron microscopy (SEM) (XL 30; Philips, Eindhoven, the Netherlands). The NP suspensions were stabilized on an aluminum disk (Marubeni, America) and dried at room temperature. The dried NPs were covered with a thin layer of gold metal using a sputter coater (SCD 005; Bal-Tec, Balzers, Switzerland).

Complex formation between CS NPs and pDNA
CS/pDNA complexes were developed at N/P ratios of 0.1, 0.5, 1, 2, 4, 8, and 16 by adding the pDNA solution to the

prepared CS NP's solution. The solution was slowly pipetted and vortexed for 3–5 s to commence complex formation and was left for 1 h at room temperature to entirely establish the CS/DNA complexes.

The binding of pDNA with CS NPs was confirmed by electrophoresis. Samples with different N/P ratios, ranging from 0.1 to 16, were electrophoresed on to 0.8% agarose gel in Tris–acetate/EDTA (TBE) buffer at 40 V for 60 min. Then, gels were stained with ethidium bromide and visualized with a UV transilluminator (Cleaver, Germany).

DNA release from CS NPs

In vitro release of DNA from CS NPs was studied by centrifugation at 4°C, 14,000 g for 30 min, after which the complex was resuspended in 800 ml phosphate-buffered saline (PBS, pH 7.4) and incubated at 37°C under 100 rpm stirring. At each marked time point, the mixture was centrifuged at 14,000 g for 30 min and DNA content in the supernatant was measured spectrophotometrically at 260 nm (Yuan et al. 2009).

In vitro transfection study

The *in vitro* transient transfection studies were performed in Chinese hamster ovary (CHO) (Pasteur Institute of Iran) cell line. The CHO cell line was passaged two days before the experiment to promote good proliferation and cell physiology. Low passage CHO cells (not more than 30 passages) were cultivated 24 h into 24-well plates at a density of 5×10^4 cells/cm^2 in 1ml of growth medium (RPMI-1640 containing 10% FBS, complemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). Before transfection, the medium was removed and the cells were washed with phosphate-buffered saline (PBS, pH 7.4), and then replaced with 150 µl of fresh culture medium without FBS. The cells were incubated with 100 µl of the CS/pDNA complexes containing 5 µg of pDNA and maintained 24 h at 37°C under 5% CO_2 (Weecharangsan et al. 2008). Cells transfected with lipofectamine and naked plasmid were used as controls. After transfection, the medium was replaced by 1 ml of fresh growth medium, and the cells were maintained for 24 h at 37°C under 5% CO_2. All transfection studies were performed in triplicate and GFP expression in transfected cells was analyzed using fluorescence microscopy.

Cytotoxicity studies

Cytotoxicity evaluation was performed with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) analysis. CHO cells were cultivated on 96-well plates at a density of 5×10^4 cells/well in 150 µl fresh culture medium supplemented with 10% FBS. Prior to transfection, the medium was removed and the cells were washed with PBS, and then supplied with 15 µl of fresh culture medium without FBS. CS and CS–pDNA suspensions (100 µl) were then added at various concentrations (N/P ratios of 0.1 to 16), respectively. After 6 h of incubation, the medium was changed with 150 µl fresh culture medium containing 10% FBS. Cell viability was determined using MTT after 72 h incubation by measuring absorbance at 570 nm. The viability of nontreated control cells was arbitrarily determined as 100%.

SDS-PAGE and western blotting

The expression of HPV-16 E7 proteins from CS–pDNA complexes in CHO cells was performed by SDS-PAGE and western blot analysis (Ghaemi et al. 2011). In SDS-PAGE analysis, the extracted total proteins lysed in SDS-PAGE sample loading buffer, and lysates separated by SDS-PAGE, and staining by coomassie blue R250. For blotting separated proteins by SDS-PAGE, transferred onto poly-vinylidene difluoride membranes (Roche, Germany), and hybridized with the monoclonal HPV-16 E7 mouse antibody (Abcam, UK), and detection performed with goat anti-mouse secondary antibody conjugated to alkaline phosphate as in secondary antibody solution. Color was developed by incubating the membrane in Di Amino Banzedin (DAB).

Statistical analysis

Statistical analysis of cell viability and *in vitro* release of the plasmids were performed using Student's t-test. A value of *P* less than 0.05 was considered to be significant.

Results

Preparation and characterization of NPs

To obtain the desired size range for NPs, low molecular weight CS polymers were necessary; hence, an oxidative depolymerization method was applied. Molecular weight
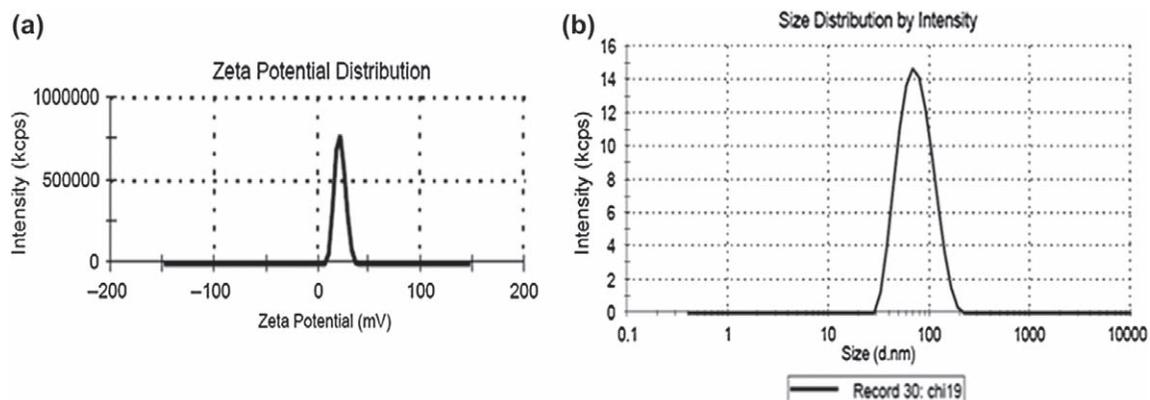

Figure 2. (a) Zeta potential of prepared nanoparticles and (b) size distribution of prepared nanoparticles both obtained from nanosizer.
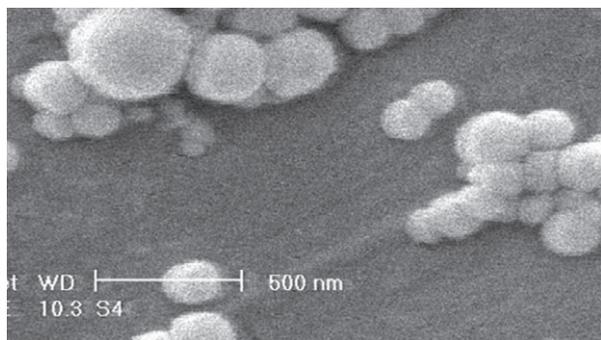
Figure 3. Scanning electron microscopy of prepared nanoparticles. Morphology of nanoparticles is spherical with smooth surface.

of depolymerized CS (12 kDa) was determined using GPC using pullulan standards.

Mean hydrodynamic diameter and zeta potential of prepared NPs are shown in Figure 2. NPs had sizes ranging from 40 to 150 nm. A narrow size distribution (PDI: 0.149) could be seen with a mean diameter of about 70 nm. The mean zeta potential of NPs was approximately + 20 mV.

Figure 3 shows a SEM image of the CS NPs containing gene. Analysis of the images shows prepared NPs had spherical-shaped structures with smooth surfaces (Figure 3).

Gel retarding analysis

The complex formation between CS NPs and pDNA at different N/P ratios (0.1 to 16) was analyzed by 0.8% agarose gel electrophoresis in TBE buffer along with naked pDNA as control. Sample was analyzed using electrophoresis at 40 V for 60 min. Figure 4 showed the naked pDNA could migrate in gel, while the pDNA loaded in CS NPs was immobile and remained in the loading wells when N/P ratio was above 1, which illustrated the complete complexation of pDNA with CS in the NPs (Figure 4).

In vitro release study

To evaluate the stability of CS/pDNA, the release behavior of pDNA from NPs in 100 h time duration at different time points was studied and measured via UV spectrometry. The results are shown in Figure 5. As can be seen in the
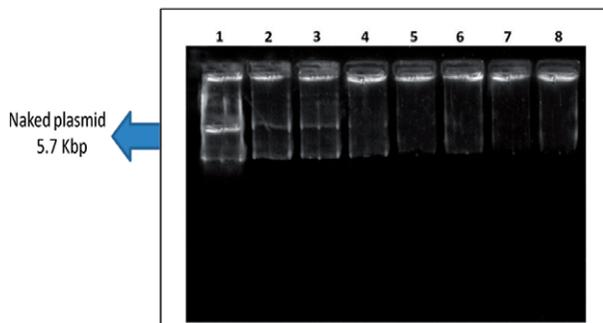

Figure 4. Agarose gel electrophoresis analysis of CS/pDNA complexes to determine complex formation. Sample were analyzed in 0.8% agarose gel by electrophoresis at 40 V for 60 min. Lane (zur Hausen 1996) pDNA plasmid; lanes (Bosch et al. 1995, Melief et al. 2000, Duggan et al. 1998, Chen et al. 2000, Somia and Verma 2000, Pouton and Seymour 2001, Ferber 2001) CS/pDNA complexes at N/P ratios of 0.1, 0.5, 1, 2, 4, 6, 8 and 16, respectively.
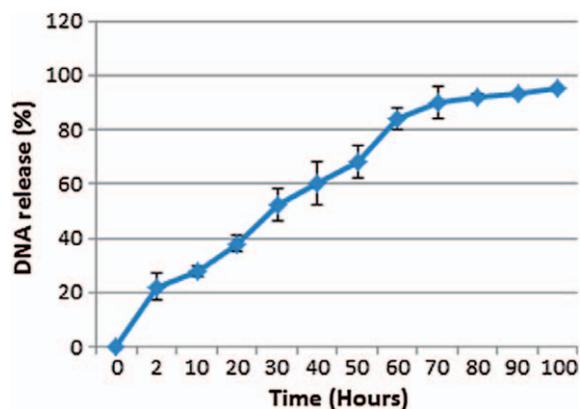

Figure 5. *In vitro* release of pDNA from chitosan nanoparticles in PBS (PH 7.4). At each marked time point, the mixture was centrifuged at 14,000 g for 30 min and DNA content in the supernatant was measured spectrophotometrically at 260 nm (Mean $\pm$ S.D, $n = 3$).

figure, the NPs could keep most of their content in the dissolution medium in early stage of insertion in the medium which simulates condition of the *in vivo*. About 90% of the content was released in 70 h.

In vitro transfection study of CS NPs

The *in vitro* transfection study was performed using CHO cell line and EGFP-N1 as marker gene. EGFP expression in cells investigated through fluorescence microscopy. EGFP expression was observed in CHO cells transfected with CS–pEGFP NPs in a similar extent to the control cells which were transfected with lipofectamine. This indicates that CS NPs can be transfected and express green fluorescent protein in mammalian cells. The results are shown in Figure 6.

Cytotoxicity of CS NPs on CHO cells

Cytotoxicity evaluation was studied to determine the safety of prepared NPs as a carrier for DNA. The percentage of viable cells was determined using an MTT reduction assay. In this experiment, the viability of nontreated control cells was considered as 100% and the viability of the NPs prepared through CS/pDNA complex at various N/P ratios (0.1 to 16) was measured in CHO cells and expressed as percentage of the viable cells to control.

As shown in Figure 7, 72-h prolonged stimulation with 0.1 to 16 N/P ratio caused a decrease in the viability of the cells. On the other hand CHO cells in a non-dose-dependent manner caused slightly decrease in cell viability. Around 28% of CHO cells suffered cell death at treatment of N/P ratio of 8 and 20% of them died at N/P ratio of 0.5 in comparison with untreated cell. However cells treated with naked pDNA had suffered from death up to 15%. Regarding these observations (comparison between results obtained from naked pDNA and NPs containing pDAN), cells treated with NPs have reserve about 85% of their viability in comparison with cells being in contact to naked pDNA, one can conclude acceptable safety for prepared NPs.

SDS-PAGE and western blot

To evaluate the expression of HPV-16 E7 gene in the CHO cells, western blot analysis using monoclonal mouse anti-HPV E7 antibody was used.
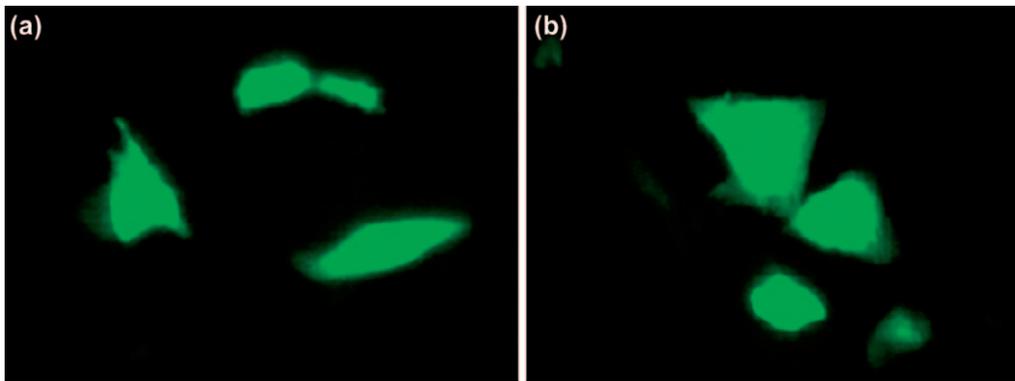
Figure 6. EGFP expression in CHO cell lines transfected by (a) Nanoparticles and (b) Lipofectamine. Cells were analyzed 48 h after transfection, and green fluorescence could be seen in cells treated with CS/pEGFP and lipofectamine.

SDS-PAGE and western blot analysis were performed on total extracted proteins, 72 h after transfection. This analysis showed an expression of HPV-16 E7 11 kDa from CS NPs (Figure 8). Transfected CHO Cell lysate with naked pDNA and lipofectamine was used as control (lane 1 and 2, respectively).

Discussion

In this study, CS NPs were prepared using the ionotropic gelation technique with TPP cross-linker and used as an efficient nonviral gene delivery carrier for HPV-16 E7 gene. NPs were prepared using this method which is simple, quick, economical, and requires no toxic solvents compared to those of the other methods (Hallaj-Nezhadi et al. 2011). In the present study intended to produce NPs of under 100 nm diameter to facilitate the entrance of the particles to the cells. The resultant NPs showed mean diameter of about 70 nm with spherical shape and a narrow size distribution (PDI: 0.149). Besides different CS /pDNA (N/P) ratios of the preparation designed to find the optimum N/P ratio with desired conditions. It was observed that NP size was irrelevant to the applied N/P ratios, because NPs were already formed when pDNA was added. Similar observations have been reported by other studies in which, this method has been used for encapsulation of DNA (Hallaj-Nezhadi et al. 2011, Nahaei et al. 2013, Vimal et al. 2013). Lavertu et al. (2006) also reported that if the CS molecular weight is high enough (MW > 10kDa), N/P ratio would not have a significant effect on particle size. Danielsen (2004) suggested that if the CS MW is high enough to condensate the DNA, than the particle size would mostly depend on the particular properties of the DNA molecule.

Very high loading efficiency yield was further observation that was found during preparing NPs with this method. This can be explained by the presence of high positive charge on the surface of NPs (+ 20 mV) (Nahaei et al. 2013) which attracts negatively charged pDNA on the surface. In previous studies, it was discovered that addition of tripolyphosphate for preparing NPs provides well-condensed and very stable NPs (Yousefpour et al. 2011). Tripolyphosphate is negatively charged, therefore it remains into CS polymers and this may explain the high loading efficiency of prepared NPs. If NPs were not stable enough, CS molecules would expel from the particles because of the same electric charge repulsion, therefore making less condensed and smaller NPs which have very low loading efficiency (Jayakumar et al. 2010, Mao et al. 2010). Jayakumar et al. and Mao et al. studied the inclusion of a negatively charged polymer into CS polymers and concluded that the inclusion increases stability of CS NPs and therefore increases gene delivery ability on CS as a carrier. Our findings were in accordance with their studies. To investigate the stability of NPs and their ability to successfully integrate with the pDNA, gel electrophoresis method was used. In this study, eight different N/P ratios were examined. Accordingly we observed that NP stability was highly dependent on the N/P ratio. N/P ratios above 1 had the most stabilized NPs, where, in NPs with N/P ratios below that, pDNA was easily released
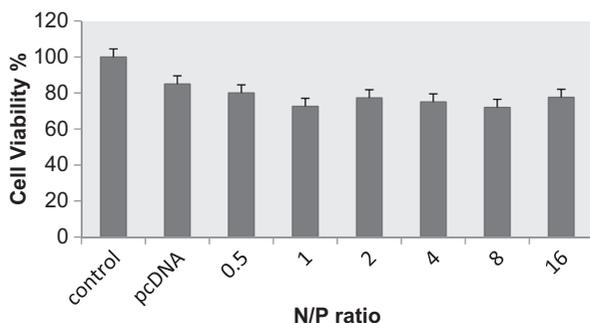

Figure 7. Cell viability assay in CHO cells. Cell viability was determined 72 h after incubation using MTT. The viability of non-treated control cells was arbitrarily determined as 100%. Results are expressed as the mean ± SD ($n = 3$).
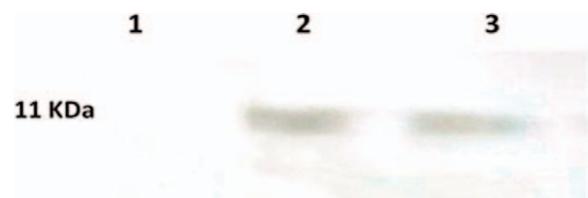

Figure 8. Western blot analyses of CHO cells infected with CS/pDNA nanoparticles. pcDNA plasmid encoding HPV-16 E7 without Chitosan nanoparticles, control (lane 1), pcDNA plasmid encoding HPV-16 E7 with lipofectamine (lane 2), pcDNA plasmid encoding HPV-16 E7 with chitosan nanoparticles (lane 3).

from NPs and migrated in the gel, therefore it was deduced that although preparing NPs with this method yields high loading efficiency it does not necessarily mean pDNA will remain on the surface of NPs. In another experiment release behavior of NPs were assessed. To have efficient gene delivery we have to make sure pDNA release from CS is optimized in a way that it does not release in blood circulation before reaching target cells and it eventually must be released from NPs inside the nucleus. Therefore, it should be considered that we must strike a balance between stability and efficient binding (Yuan et al. 2009). In this study in 70 h the cumulative drug release from NPs was approximately 90%. This simulated the *in vivo* condition and revealed that CS NPs had potential for carrying and protecting pDNA.

Another attempt was made to evaluate their transfection efficiency in CHO cells. NPs with the desired size diameter and the highest loading efficiency that had enough stability obtained from gel electrophoresis studies were chosen for testing transfection efficiency in cell culture. The human cytomegalovirus promoter of the pcDNA3.1 has been shown to be very powerful in CHO cells, therefore CHO cell line is proved to be highly efficient in transfection with genetic material, and this was previously investigated and reported by our team (Ghaemi et al. 2011). However, CHO cell line cannot be considered an ideal replacement for human cell lines in experimental studies since it does not carry all of the sugar-transferring enzymes that are present in human cell lines (Dutta et al. 2012).

To investigate transfection efficiency of NPs pEGFP was employed. pEGFP is a plasmid that produces fluorescent protein inside the transfected cells. The green fluorescent observed by fluorescent microscope is an indication of successful transfection of pEGFP inside the cells. Other studies from our team and also other articles showed little or no cytotoxic effects of CS in cell culture. Nevertheless the cytotoxicity of prepared NPs as a carrier using the MTT viability assay to investigate whether using CS as a carrier for E7 delivery poses any threat to target cells viability. In this experiment NPs produced with different N/P ratios was utilized. Results revealed no significant correlation between N/P ratio and cell viability. The viability of cells treated with NPs containing pDNA was between 70 to 80% while, the viability of untreated cells and cells treated with naked pDNA was 100% and 85%, respectively. This observation shows CS as a carrier has some undesired effects on treated cells. Therefore it demands more investigation to prepare a formulation to lessen undesired effects of CS on target cells. Western blot analysis was performed to evaluate the expression of HPV-16 E7 gene in CHO cells transfected with CS NPs. This analysis showed that HPV-16 E7 was successfully expressed from CS NPs and could be detected in the culture supernatant. Data illustrated in Figure 8 indicate that the CS NPs are able to exert expression similar to that of lipofectamine, exhibiting high transfection efficiency of CS NPs. This transfection efficiency of the vector suggests that CS NPs could be a promising nonviral gene delivery vector, which is easy to synthesize, highly efficient and has low cytotoxicity. The vector could effectively deliver exogenous HPV-16 E7 in tumor models.

Conclusion

Observations in this study revealed that CS/pDNA NPs, prepared with ionotropic gelation method with N/P ratio of 1 to 16, have provided a promising formulation for pDNA delivery. Using CS as a carrier brought an opportunity for efficient nonviral gene delivery although the efficiency of CS as a gene carrier is relatively low compared to viral gene delivery methods. However more investigation should be conducted aiming formulations less toxic with higher gene delivery capabilities.

Declaration of interest

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