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Nuclear localization signal peptides enhance transfection efficiency of chitosan/DNA complexes

Praneet Opanasopit[a], Theerasak Rojanarata[a,*], Auayporn Apirakaramwong[a], Tanasait Ngawhirunpat[a], Uracha Ruktanonchai[b]

[a] Nanotechnology for Drug/Gene Delivery Systems Group, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand
[b] National Nanotechnology Center, Thailand Science Park, Pathumthani, 12120 Thailand

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ABSTRACT

The purpose of this study was to investigate the potential of nuclear localization signal (NLS) "KPKKKRKV" to mediate the in vitro transfection efficiency of chitosan (CS)/DNA complexes, aiming at its use in gene therapy applications. In the preparation of CS/DNA complexes containing NLS, peptide with NLS was directly incorporated without covalent conjugation to pDNA or chitosan. The gene transfer efficiency of CS/DNA complexes with and without NLS was evaluated in the human cervical carcinoma cell line (Hela cells). The CS/DNA complex containing NLS increased transfection efficiencies in a NLS-dose dependent manner on the Hela cells, compared to the control (CS/DNA complex or NLS). The highest transfection efficiency was significantly observed in CS/DNA complex at the weight ratio of 8 with 120 μg NLS and was 74-fold higher than that in the cells transfected with CS/DNA complex. Cytotoxicity of the NLS/CS/DNA complexes increased as the amount of the peptide increased, however, over 80% average cell viability was observed for complexes at the effective concentration of the peptide for transfections. Therefore, the NLS is expected to be a potent transfection enhancing agent without a covalent conjugation to pDNA or chitosan. Our findings suggest that the high gene expression with the negligible cytotoxicity can be achieved by adding the NLS peptide to chitosan/DNA complexes at an optimal ratio.

1. Introduction

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. The main objective in gene therapy is successful in vivo transfer of the genetic materials to the targeted tissues. However, naked therapeutic genes are rapidly degraded by nucleases and show poor cellular uptake, so the development of safe and efficient gene carriers is one of the prerequisites for the success of gene therapy (Rolland, 2005). One approach is a non-viral delivery system based on supramolecular assembly. Cationic lipids and cationic polymers have been employed as non-viral gene transfer agents. These cationic substances form complexes with anionic DNA by electrostatic interaction. The resultant cationic DNA complexes were taken up by cells through electrostatic interaction because the cell surface is negatively charged. Among those, cationic liposomes are widely used for almost all animal cells because they have nonspecific ionic interaction and low toxicity (Ruozi et al., 2003). However, there are some limitations since when they are used for in vivo transfection, they are unstable. Therefore, many polymeric cationic systems such as polyethyleneimine (PEI), cationic peptides (poly L-lysines; PLL), cationic dendrimers and chitosan have been studied for in vitro as well as in vivo application (Tang et al., 2006; Tiera et al., 2006).

Chitosan (CS) is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by alkaline deacetylation of chitin. CS is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values, but soluble in acidic medium. CS is biocompatible, biodegradable and non-toxic; therefore, it has been proposed as a safer alternative to other non-viral vectors (Ishii et al., 2001; Weecharangsan et al., 2006). Formulation parameters such as molecular weight (MW), degree of deacetylation (DD), N/P ratio (ratio of positively charged chitosan to negatively charged DNA), and pH of transfection medium were found to affect the transfection efficiency of CS/DNA complexes (Huang et al., 2005; Lavertu et al., 2006; Weecharangsan et al., 2008). The main drawback of CS is the poor water solubility at physiological pH and low transfection efficiency. Several CS derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics. Modified CS such as glycol CS or PEGylated CS and quaternized CS, low MW soluble CS, and salt forms could be

* Corresponding author. Tel.: +66 34 255800; fax: +66 34 255801.
E-mail address: teerasak@su.ac.th (T. Rojanarata).

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possible ways to circumvent the solubility issues. To improve gene transfection, chemically modified CS was reported (Kim et al., 2003; Chae et al., 2005; Lee et al., 2007; Opanasopit et al., 2008, 2009).

Recent studies indicated that the nuclear membrane is a serious barrier to the delivery and expression of exogenous gene and thus many attempts have been made to overcome this barrier. Among various approaches, the use of nuclear localization signal (NLS) peptides for non-viral gene transfer has been widely investigated (Dean et al., 1999; Escriou et al., 2003; Prasad and Rao, 2005). Nuclear pore complexes (NPCs) control the passage of molecules in and out the nucleus. The inner pore of a NPC allows free, passive diffusion of molecules of up to 9 nm in diameter. Additionally an active transport of small molecules up to 25 nm is possible (Ludtke et al., 1999). Larger molecules require so-called NLS that are recognised by cytoplasmatic transport receptors and mediate the nuclear uptake. NLS sequences are typically less than 12 residues in length and rich in basic amino acids resulting in a net positive charge. The most studied and therefore best known NLS sequences are peptides derived from viruses like Tat (transactivating) protein or *Antennapedia homeodomain* protein derived ones, but arginine/lysine-rich NLS such as for the simian virus 40 (SV40) large T antigen (PKKKRKV) seem to be far more efficient than those peptides. The various approaches explored differ mainly in the method used for the covalent (Ludtke et al., 1999; Prasad and Rao, 2005; Bremner et al., 2004) or non-covalent (Chan and Jans, 1999; Cartier and Reszka, 2002) attachment of the NLS peptide(s) to DNA and in the use of linear DNA or circular (plasmidic) DNA. In the case of NLS covalently attached to the DNA, care has to be taken to avoid binding of the NLS within the expression cassette, hence blocking subsequent transcription of the transgene. While many of these approaches have met with limited success, a significant (10–1000-fold) enhanced gene expression was obtained following ligation of a NLS-oligonucleotide conjugate to one or both ends of a linear DNA (Zanta et al., 1999). Other approach of NLS covalent attachment is to attach with gene carriers. Jeon et al. showed that the covalent conjugation of a NLS (SV40 peptide) on poly(L-lactide-co-glycolide) nanospheres enhanced the gene transfection efficiency (Jeon et al., 2007). Recently, NLS was chemically conjugated to a DNA intercalating reagent. Yoo et al. revealed that the DNA/PEI complex consisting NLS attached to psoralen, a nucleic acid-intercalating agent increased transfection efficiencies on COS-1 cells (Yoo and Jeong, 2007). On the other hand, Boulanger et al. reported that the expression of the transgene in most cases did not improve upon complexation of plasmid DNA with NLS-acridine conjugates prior to its formulation as lipoplexes or polyplexes (Boulanger et al., 2005).

The ionic residues of NLS-containing peptide can also interact with the polycations typically used for DNA condensation or the hydrophobic residues with lipid components. The NLS can therefore influence the physical properties of the heteroplex such as particle size, DNA condensation rate, surface charge distribution and intracellular stability, which eventually influence the transfection efficiency. In this study, we determined physicochemical properties of CS/DNA complexes such as particle size and charge with and without NLS. A number of variables that influenced transfection efficiency such as CS/DNA N/P ratio, amount of NLS, pH of culture medium and serum were investigated. Based on these data, we elucidated the main factors influencing the enhanced transfection efficiency in vitro.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Seafresh Chitosan Lab., Thailand with MW of 45 kDa and 87% degree of deacetylation. Chitosan salt (CS; chitosan HCl) was prepared as previously described (Weecharangsan et al., 2008). Briefly, chitosan was dissolved in distilled water containing hydrochloric acid at 1:1 molar ratio. The solution was stirred for 12 h and spray-dried. Polyethylenimine (PEI), MW 25 kDa, was purchased from Aldrich, Germany. The NLS peptide (KPKKKRKV) was synthesized by Peptide Synthesis Department, Bio Basic Inc. Ontario, Canada. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen, USA. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, USA. The λHindIII were obtained from Promega, USA. Huh7 (Human hepatocellular carcinoma) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

3. Methods

3.1. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra®, Göttingen, Germany). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate-EDTA (TAE) buffer, pH 8.0 using λDNA/HindIII as a DNA marker.

3.2. Preparation and characterization of CS/DNA complexes with and without NLS

The CS/DNA complexes were prepared at various N/P ratios by adding the DNA solution to the CS solution. The mixture was gently mixed using pipette for 3–5 s to initiate complex formation and then NLS solution was added and left for 15 min at room temperature. The complex formation was confirmed by electrophoresis. Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/ml). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μl of CS derivatives/DNA complex containing 1 μg of DNA.

3.3. Size and zeta potential measurements

The particle size and surface charge of CS/DNA complexes with and without NLS were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior used. All samples were measured in triplicate.

3.4. Morphology

The morphology of the CS/DNA complexes was determined by Atomic Force Microscope (AFM, SPI4000-SPA400, Chiba, Japan) using tapping-mode AFM in air. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior used. These samples were dropped immediately onto freshly cleaved mica and dry in air.

3.5. In vitro transfection

Hela cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 ml of growth medium (DMEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with 0.5 ml of the CS/DNA complexes with and without NLS at various N/P ratios containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

3.6. Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. Hela 7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μl of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to transfection, the medium was removed and the cells were rinsed with PBS, and then supplied with the CS/DNA complexes with and without NLS in the same concentrations as in vitro transfection experiment. After treatment, CS/DNA complexes solutions were removed. Finally, the cells were incubated with 100 μl MTT containing medium (1 mg/ml) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

3.7. Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

4. Results and discussion

4.1. Characterization of CS/DNA complexes with and without NLS

In order to determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between CS and DNA at different CS concentrations with and without NLS. The formation of complexes between CS and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. By varying the concentration of CS and fixing the DNA concentration, ratios of the weight of positively charged CS (due to amine groups) to negatively charged DNA (due to the phosphate groups), which were referred as weight ratio of the particle formulations were varied. Fig. 1 shows DNA marker (lane 1), the naked DNA (lane 2) and CS/DNA complexes at weight ratios of 0.5, 1, 2, 3, 4, 8 and 16 (lanes 3–9). The naked DNA showed the DNA bands, whereas complexed DNA was retained within the gel loading well for CS at weight ratio above 2, illustrating that complete CS/DNA complexes were formed. In contrast to the preparation of CS/DNA complexes containing 120 μg NLS peptides, it was found that complexes could be completely formed at all ratios. These indicated that the binding between CS and DNA was dependent on NLS. NLS sequences are rich in basic amino acids resulting in a net positive charge that facilitated the complexation between CS and DNA (Bremner et al., 2004).

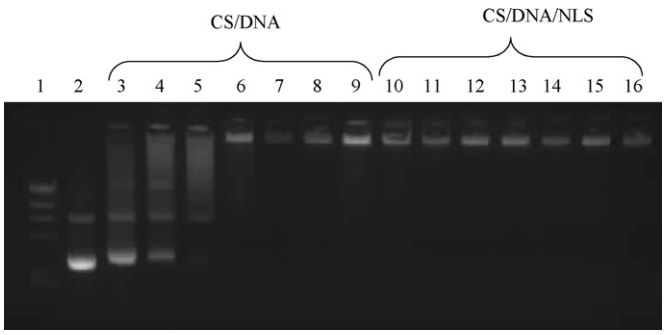

Fig. 1. Gel retarding analysis of CS/DNA complexes formulated with and without NLS. Lane 1, DNA marker; lane 2, pEGFP-C2 plasmid, lanes 3–9 (CS/DNA complexes) and lanes 10–16 (CS/DNA complexes with 120 μg of NLS) at weight ratios of 0.5, 1, 2, 3, 4, 8 and 16, respectively.

Particle size and the zeta potential of various weight ratios of CS/DNA complexes with and without NLS were shown in Table 1. The particle size of CS/DNA complexes slightly increased with the increasing weight ratio from 2 to 24 in the range of 300–500 nm. The zeta potential of the complexes was found to increase with the increase in weight ratios of CS/DNA complexes due to their higher density of protonated amines in the CS backbone. A similar result was observed in CS/DNA with NLS. At low amount of CS (2–4 μg), an addition of NLS resulted in increase in the particle size. On the other hand, at the higher amount of CS (8–24 μg), an addition of NLS resulted in decrease in the particles size. Moreover, the addition of NLS yielded the increase in the zeta potential. These results indicated that an addition of NLS affected both particle size and surface charge of CS/DNA complexes. The morphological examination of the CS/DNA complexes at a weight ratio of 4 with 120 μg NLS was performed by AFM. The AFM images (Fig. 2) revealed that the complexes were spherical with nanosize.

4.2. In vitro transfection

The achievement of high gene transfection efficiency is a final goal for the development of novel gene carriers. To investigate the NLS mediated gene transfection efficiencies, in vitro gene transfection assay was performed with Hela cells using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). In order to investigate the optimal conditions for gene transfection, CS/DNA complexes

Table 1
Particle size and zeta potential of CS/DNA complexes with and without NLS.

CS (μg)	NLS (μg)	DNA (μg)	Particle size (nm)	Zeta potential (mV)
0	120	1	ND	ND
2	0	1	332 ± 27	3.7 ± 1.5
	120	1	603 ± 25	10.4 ± 1.5
4	0	1	410 ± 12	6.2 ± 2.5
	60	1	542 ± 7	12.1 ± 5.5
	120	1	495 ± 41	11.9 ± 5.1
	160	1	639 ± 27	11.2 ± 3.4
	200	1	580 ± 50	13.4 ± 1.9
	240	1	676 ± 26	15.6 ± 0.7
8	0	1	423 ± 18	8.1 ± 1.6
	120	1	408 ± 26	13.6 ± 3.1
16	0	1	507 ± 17	8.4 ± 0.8
	120	1	255 ± 14	14.3 ± 3
24	0	1	397 ± 40	9.3 ± 1.7
	120	1	185 ± 6	13.6 ± 3.2

ND = not determined.

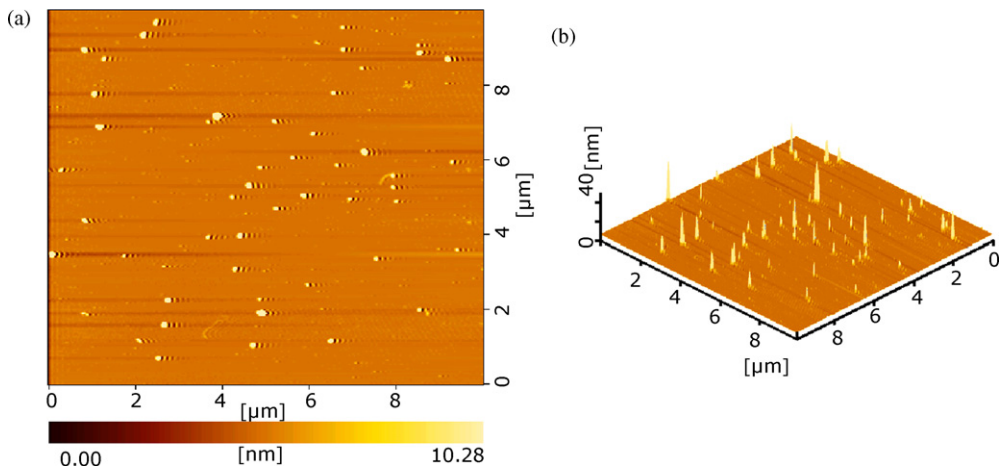

Fig. 2. The atomic force microscope (AFM) images of CS/DNA complexes at weight ratio of 4 mixed with 120 μg of NLS: (a) planar image and (b) three-dimensional image.

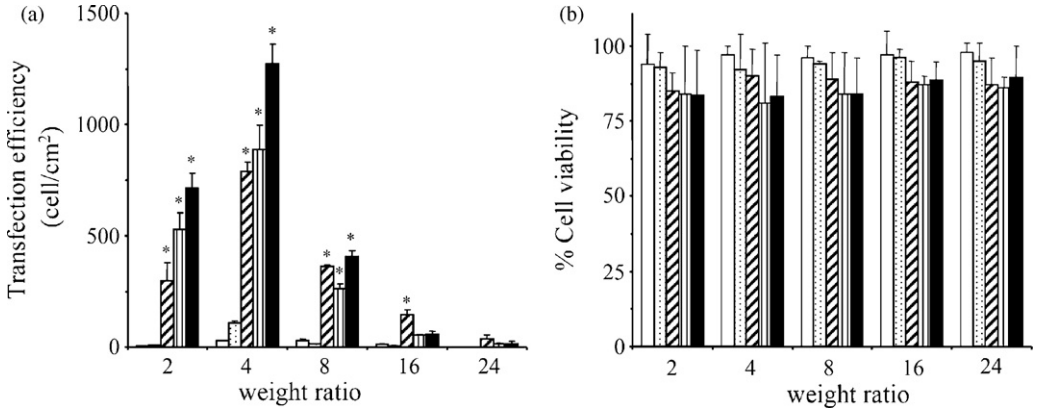

Fig. 3. (a) Transfection efficiencies and (b) cell viability of CS/DNA complexes at varying N/P ratios formulated with NLS: 0 μg (□), 30 μg (⬚), 60 μg (▨), 90 μg (▥) and 120 μg (■) in Hela cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

were formulated with various weight ratios (2, 4, 8, 16 and 24) with and without NLS. Polyethylenimine (PEI, 25 kDa) complexed with DNA at the weight ratio of 1 was used as a positive control and had the gene transfection of 600 cell/cm^2. In all studies, there were no transfection in control (cells without complexes), naked DNA and NLS/DNA complexes with various weight ratios (30, 60, 90, 120 and 240). As shown in Fig. 3 (at pH 6.2), the gene transfection efficiencies were significantly influenced by the CS/DNA ratios and amount of NLS. By increasing the ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. The highest transfection efficiency was observed at the weight ratios of CS/DNA complex of 4 with the addition of 120 μg NLS (Fig. 3a). Fig. 4a represents the influence of NLS amount (0–240 μg) on transfection efficiency of CS/DNA complex at the weight ratio of 4. The transfection efficiencies increased when the amount of NLS increased and reached the maximum at 120 μg. Then, the transfection efficiencies decreased by further increment of the NLS. These results revealed that not only the CS/DNA complex ratio but also the amount of NLS affected the gene transfection efficiency. Our results clearly demonstrate that ternary complexes,

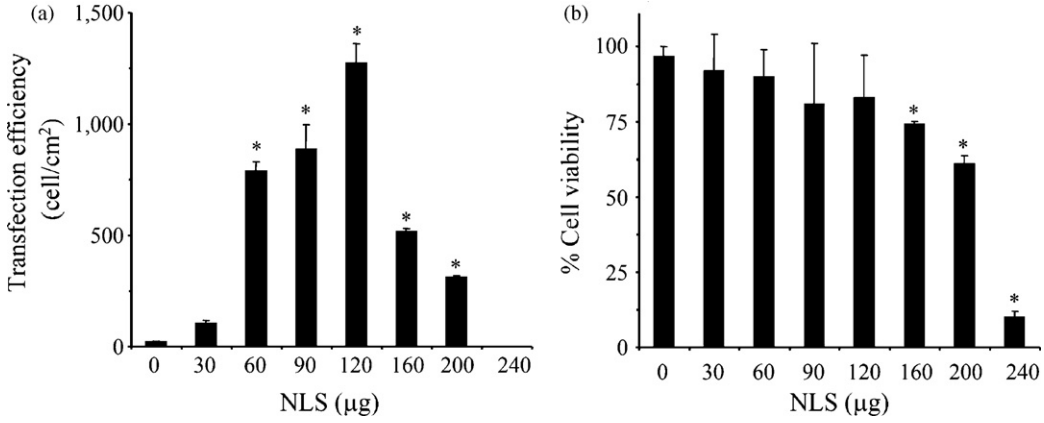

Fig. 4. (a) Transfection efficiencies and (b) cell viability of CS/DNA complexes at weight ratio of 4 formulated with various amount of NLS in Hela cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

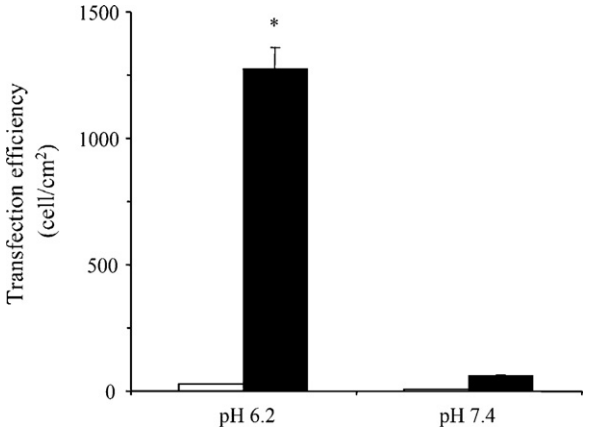

Fig. 5. Effect of pH medium at pH 7.4 and pH 6.2 with NLS 120 μg (■) and without NLS (□), on transfection efficiencies of CS/DNA complexes at the weight ratio of 4 in Hela cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

resulting from the association of NLS to CS/DNA complexes, are significantly more efficient in mediating transfection than the corresponding NLS/DNA or CS/DNA complexes. Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). As shown in Fig. 5, pH dramatically affected both CS and CS with NLS with decreasing the transfection efficiency by increasing pH values from 6.2 to 7.4. Although the exact mechanism of NLS mediated efficient gene delivery remains to be further studied, our results clearly demonstrate that the addition of NLS could thereby influence the physical properties of the heteroplex such as particle size, surface charge distribution, DNA condensation, and intracellular stability. These effects could influence the transfection efficiency of the complex and could be potential candidate for non-viral gene carriers.

4.3. Effect of NLS/CS/DNA complexes on cell viability

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that CS were less toxic than other cationic polymers such as poly-lysine and PEI in vitro and in vivo (Lavertu et al., 2006). However, the toxicity of association of NLS to CS/DNA complexes was not studied. Therefore, the cytotoxicity study of NLS/CS/DNA complex was conducted in Hela cells. Fig. 3b shows the effect of NLS/CS/DNA complex on cell viability. There was significant decrease in cell viability when Hela cells were incubated with increasing amount of NLS in all CS/DNA ratios. However, the viability was over 80% at the addition of 120 μg NLS where the highest transfection efficiency was obtained. By further increment of the NLS more than 120 μg, % cell viability was dramatically decreased (Fig. 4b) resulting in decrease the transfection efficiency. A similar result was observed in previous studies (Trabulo et al., 2008).

5. Conclusion

Our results clearly demonstrate that ternary complexes, resulting from association of NLS to CS/DNA complexes, are significantly more efficient in mediating transfection than the corresponding NLS/DNA or CS/DNA complexes. Cytotoxicity of the NLS/CS/DNA complexes increased as the amount of the peptide increased, however, over 80% average cell viability was observed for complexes at the effective concentration of the peptide for transfections. Our findings suggest that the high gene expression can be achieved by adding NLS peptide to CS/DNA complexes at optimal ratio.

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