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ORIGINAL RESEARCH PAPER

Optimization of conditions for gene delivery system based on PEI

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ABSTRACT

Objective(s): PEI based nanoparticle (NP) due to dual capabilities of proton sponge and DNA binding is known as powerful tool for nucleic acid delivery to cells. However, serious cytotoxicity and complicated conditions, which govern NPs properties and its interactions with cells practically, hindered achievement to high transfection efficiency. Here, we have tried to optimize the properties of PEI/ firefly luciferase plasmid complexes and cellular condition to improve transfection efficiency.

Materials and Methods: For this purpose, firefly luciferase, as a robust gene reporter, was complexed with PEI to prepare NPs with different size and charge. The physicochemical properties of nanoparticles were evaluated using agarose gel retardation and dynamic light scattering. MCF7 and BT474 cells at different confluency were also transfected with prepared nanoparticles at various concentrations for short and long times.

Results: The branched PEI can instantaneously bind to DNA and form cationic NPs. The results demonstrated the production of nanoparticles with size about 100-500 nm dependent on N/P ratio. Moreover, increase of nanoparticles concentration on the cell surface drastically improved the transfection rate, so at a concentration of 30 ng/μ, the highest transfection efficiency was achieved. On the other side, at confluency between 40-60%, the maximum efficiency was obtained. The result demonstrated that N/P ratio of 12 could establish an optimized ratio between transfection efficiency and cytotoxicity of PEI/plasmid nanoparticles. The increase of NPs N/P ratio led to significant cytotoxicity.

Conclusion: Obtained results verified the optimum conditions for PEI based gene delivery in different cell lines.

Keywords: *Polyethyleneimine(PEI), Gene delivery, Nanoparticles, Transfection*

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INTRODUCTION

Genetic manipulation of mammalian cells is currently used in the wide area of biomedical science and translation medicine. Recent advances in molecular genetics have led to emerging of numerous types of genetic modification tools including plasmid, siRNA, PNA, microRNA, Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 which have been used in many studies for modification of genetic and transcription behavior of cells [1-4]. Indeed mentioned tools can be used for the screen nucleic acid based-drugs, production of the disease model, the study of metabolic pathways, production of recombinant protein, development of whole-cell biosensor [5], cell therapy and engineering immune cells [6-8].

Nevertheless, some of the barriers including, cell membrane, endosome entrapment, and serum nucleases are mainly reduced the efficiency of genetic modification tools. For this purpose, viral and non-viral carriers can be used for gene transfection. Although viral carriers can transfect cells with high efficiency, their preparation is also time-consuming and high cost. More important, these carriers are

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unable to transfer of siRNA and other novel systems like CRISPR/Cas into cells. Therefore, various non-viral materials have been developed as synthetic nucleic acid delivery carriers [9]. These carriers are including, different synthetic and natural compounds such as calcium phosphate, poly-L-Lysine, polyethyl-eneimine(PEI), dendrimer, protamine, and lipofe-ctamine, which are able to surpass the mentioned barriers. Among the mentioned carrier branched PEI, owing to proton sponge capacity, is known as a high efficient transfection agent [10]. Indeed, protonation of primary, secondary and tertiary amino groups provide a high buffering capacity for PEI, which leads to lyse of endosome and release of the cargo into the cytoplasm [11].However, due to complex condition which governs the physicochemical properties of PEI/ DNA nanoparticles and its interaction with the cell, this polymer failed to transfect different cells properly [11]. Therefore, it is an urgent need to optimize the transfection process for PEI. So far, RT-PCR, western blotting and gene reporters such as Green Fluorescent Protein and firefly luciferase have been used for monitoring transfection process and optimization of gene delivery nanoparticles [12, 13].

Among mentioned system luciferase as gene reporters make fast and robust signal following to transfection process [5,14,15]. Therefore, we have used luciferase plasmid, *psiCHECK*, in complex with PEI to optimization the transfection of different human cell lines. This study aimed to optimize the transfection process of PEI/psiCHEK nanoparticles using the light production of luciferase enzyme.

For this purpose, we have optimized the transfection condition and analyzed the role of a series of important factors including, size and charge of nanoparticles, concentration of nanoparticles on the cell medium, time of transfection, cellular number per area and finally the effect of cytotoxicity on transfection efficiency of PEI/plasmid based nanoparticles.

MATERIALS AND METHODS

Materials

High molecular weight (25000 Da), anhydrous branched PEI Sigma–Aldrich.**,** *psiCHECK* (Promega), fetal bovine serum (FBS), Roswell Park Memorial Institute medium (RPMI), Dulbecco's Modified Eagle Medium (DMEM), MTT and Agarose were purchased from Gibco.

Cell lines

MCF7 and BT 474 Cells were obtained from Pasteur Institute of Iran and cultivated in DMEM and RPMI medium supplemented with 10 % fetal bovine serum and 100 U/ml penicillin and 100 g/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2/95 % O_2.

Preparation of nanoparticles

In this study, nanoparticles were prepared by mixing of PEI 25KD with the psiCHECK plasmid. Briefly, 1µg of DNA was mixed with different amounts of PEI according to N/P calculation. N/P ratio refers to the molecular ratio of amines (N; cationic groups) in PEI to phosphates (P; anionic groups) in nucleic acids. N/P ratio was calculated using the molar ratio of positive charge to negative charge.

$$N/P = \frac{\frac{\mu g\ vector}{Mw\ of\ \text{repeated unit}}}{\frac{(number\ of\ positive\ charge)}{\frac{\mu gDNA}{330}}}$$

The molecular weight of a repeated unit of PEI is 473 Da with four amine groups:

$$N/P\ = \frac{\frac{\mu g\ \text{PEI}}{\frac{Mw(473)}{(4)}}}{\frac{\mu gDNA}{330}}$$

To obtain an N/P ratio of 12 for 1µg DNA and 4.2 µg of PEI were mixed directly or diluted in 100 µl of PBS buffer. Then the mixtures were incubated at 25 °C at different times including 5-30 minutes.

Characterization of nanoparticles

Gel retardation

The DNA binding ability of PEI at various N/P ratios was examined using gel retardation assay. Briefly, 1µg of pDNA was mixed with PEI at different N/P ratio. The nanoparticles were loaded on agarose gel and electrophoresed for one hour. The nanoparticles were visualized using ethidium bromide staining, followed by visualization with UV illumination (UV Tech, Germany).

Size and zeta potential analysis of nanoparticles

The average particle size and size distribution of the complexes were determined by dynamic light

scattering using photon correlation spectroscopy. Zetasizer Nano ZS instrument (Malvern Instruments, UK) was used to measure both particle size and zeta potential at 25 °C. Nanoparticles were prepared by addition of PEI (1mg/mL) to the DNA and incubating for 15 minutes at RT.

Transfection

Cells were seeded in a 12-well plate (SPL Life Sciences) 24 h prior to transfection. Serum-free media was added to each well for 2 h. In order to optimization of transfection in terms of nanoparticles physicochemical properties and cellular conditions following experiments were performed:

A) The nanoparticle at different N/P ratio with 1µg DNA was prepared once confluency of 70% was obtained the medium was replaced with DMEM medium plus nanoparticles at different N/P ratio after 4 hours the complete medium was added to cells. B) In the other approach, MCF7 and BT474 cells were prepared at various cell number/areas (confluency) were prepared at transfection time and then the medium over the cells was replaced with medium plus nanoparticles. C) In the other approach, the nanoparticles at different concentration were used for transcription. Once a confluency of 70% was achieved the medium was replaced with the various volumes of medium, supplemented with nanoparticles, and then the complete medium was added to the cells. Finally, cells were lysed using CCLR buffer or hypotonic buffer. The luciferase activity was assessed in the presence of luciferin, ATP, and Mg^{2+} with a luminometer (Berthold Detection Systems, GmbH).All mentioned different condition for transfection optimization is summarized in Table 1.

Cytotoxicity Assay

The cytotoxicity is one of the important aspects should be considered for delivery and transfection material. The cytotoxicity of PEI/*psiCHECK* nanoparticles was evaluated using MTT assay. 10^4 cells were seeded in 96-well plates prior to the experiment. At treatment time, the medium of cells was replaced with fresh medium supplemented with nanoparticles contain 0.2µg of DNA at different N/P ratio (5-20). 44 hours later the MTT reagent (5 mg/ml) at a final concentration of 0.5 mg/ml was added to each well, and the cells were incubated at 37 °C for 4 h. The formazan product was dissolved in 100 µl dimethyl sulfoxide (DMSO). Color intensity was measured using a microplate reader (ELx800, Biotek, USA) at 570 nm. The result was present relative to untreated cell 100% viable. Data was reported as mean ± SD (n:3).

In a further assay, different amounts of fusion vectors were added to the cells and left for 48 h without exchanging the media. The control well was not treated, and PEI was used as a positive control.

Table1. Different tested conditions for gene transfection

Factors	Conditions
N/P Ratio	3,6,12,18,24
DNA amount	1,2 µg
Confluency	10,25,40,60,80 %
Volumes of medium	30,50,60,100 µl
Time of assay post transfection	24,48 hours

RESULTS AND DISCUSSION

Preparation of nanoparticles

Like viral vectors, synthetic material can be used for intracellular delivery of nucleic acid base molecules. Among various materials, Polyethylenimine (PEI), as a gold standard can carry different type of Nucleic acids without regard to its type and size, in a charge-dependent manner and due to its high buffering capacity can easily disrupt the endosome/lysosome's membrane.

In this study, we have used *psiCHECK* plasmid, luciferase encoding plasmid to prepare nanoparticle for gene delivery. Here, we have used N/P ratio factor to control nanoparticle properties. In the other word, ratio of negatively charged group of DNA and positively charged group of PEI used as a universal factor for the preparation of different particles. So we have analyzed the effect of this factor on the DNA binding and transfection efficiency.

At first step, we have examined the pDNA binding capacity of PEI. As shown in Fig. 1a PEI efficiently retards the electrophoretic movement of pDNA. These results were consistent with the inherent capacity of PEI for neutralizing the negative charge of pDNA owning to the formation of electrostatic bridge between the amine group of PEI and phosphate group of DNA. Moreover, weak ethidium bromide signal at high N/P ratio indicates tight binding between the nucleic acid and polymer, which resulted in ethidium bromide exclusion. On the other hand, we have analyzed the effect of incubation time on the PEI/DNA

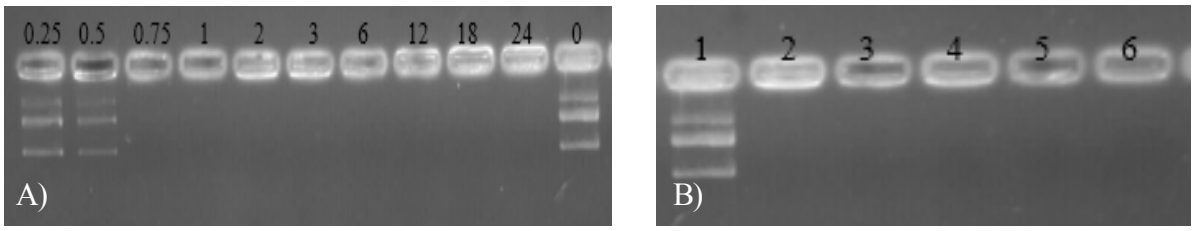

Fig. 1. Agarose gel retardation assay of PEI/psiCHECK complex and the effect of incubation time on nanoparticles formation rate A) PEI/psiCHECK nanoparticles at several of N: P ratios were prepared and loaded on the agarose gel. B) Agarose gel retardation of PEI/psiCHECK nanoparticles at N: P ratio of 12 which incubated for different time. ; Lane 1: naked plasmid, lane 2: immediately after addition of PEI, lane 3: 5 min after incubation, lane 4:10 min, lane 5: 15 min, lane 6; 20 min

nanoparticles formation. The result was demonstrated that PEI instantly bound to the DNA and neutralize its electrostatic charge (Fig. 1b). The result demonstrated that PEI can immediately form Nanoparticle in presence of DNA, which is very useful for High-throughput transfection.

Size and zeta potential analysis of Nanoparticles

Different studies have shown that size of particles has a great impact in cellular internalization[16, 17]. In this study, we have examined the effect of N/P ratio on the size of PEI/pDNA nanoparticle. Unexpectedly. As shown in Fig. 3a, DLS demonstrated the production of nanoparticles with size about 100-500 nm dependent on N/P ratio, which representative these result presented the suitable DNA condensing ability of this polymer.

The increase of PEI content significantly increased the complex size, so that at N/P ratio 24 a large particle was observed. Therefore, at this N/P ratio nanoparticles may enter to cell in a clathrin-mediated endocytosis at low N/P ratio. Furthermore, their small size proposed easier movement in the cytoplasmic environment. Unexpectedly, at higher N/P ratio, DLS disclosed a series of large particle, which may be formed as a result of aggregation (Fig. 2b) .

This result, suggested that the N/P ratio of NPs by tuning the size of nanoparticle might affect the gene transfection and level of expression.

The N/P ratio of complexes affects the size, gene transfection and level of expression of them[18].

In the other effort, we have examined the surface charge of PEI/psiCHECK nanoparticles at different N/P ratio. As shown in Fig. 3 the charge of nanoparticles was increased with the increase of N/P ratio. Indeed, all of the particles showed a positive surface charge, which is essential for interaction of nanoparticles

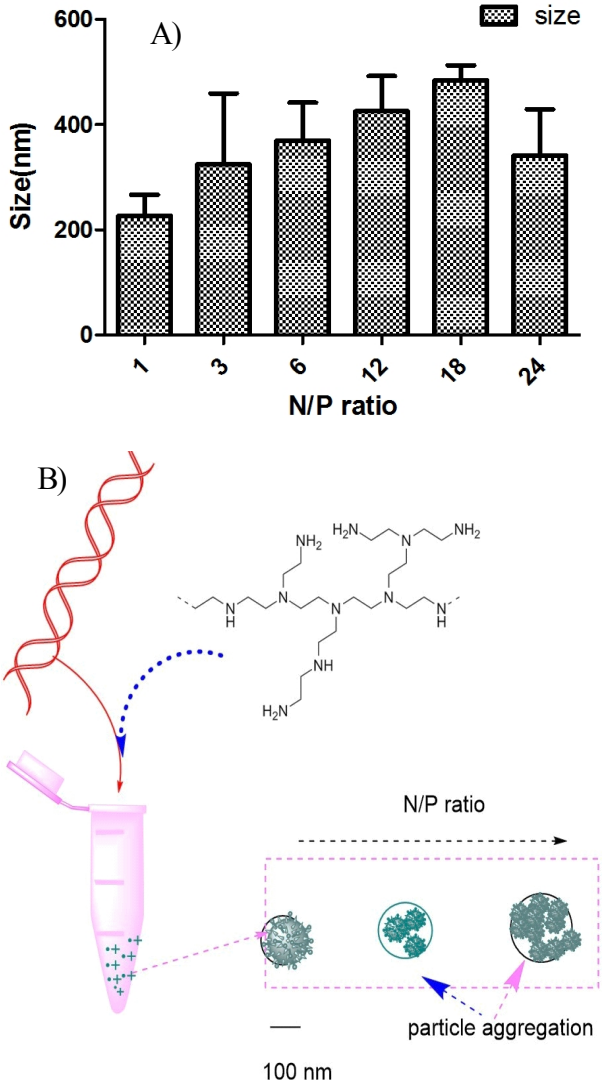

Fig. 2. A) The particle size analysis of DNA/ *psiCHECK* nanoparticles at various N/P ratios. B) Schematic representation of aggregation of NPs

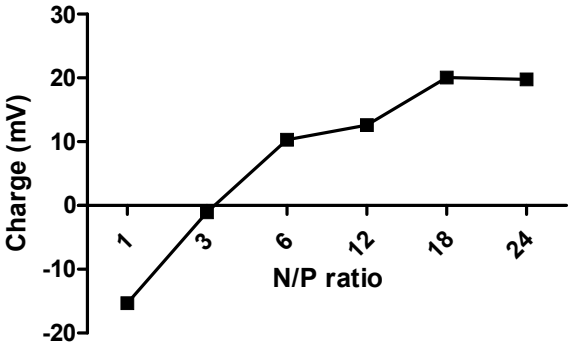

Fig. 3. The zeta potential analysis of DNA/ *psiCHECK* nanoparticles at various N/P ratios

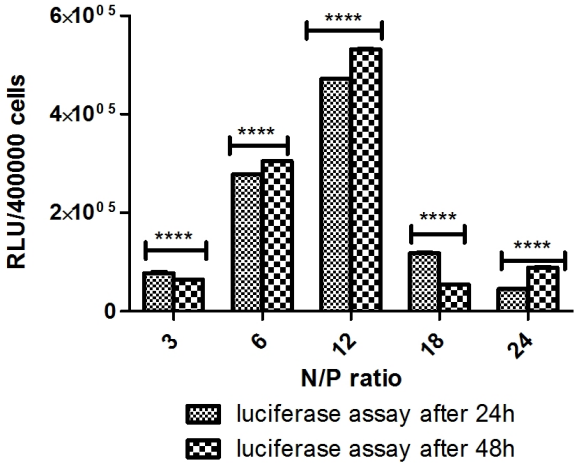

Fig. 4. The effect of N/P ratio on the transfection efficiently PEI/ psiCHECK2 Nanoparticles. The BT474 cells were seeded and transfected by PEI/*psiCHEK* Nanoparticles at different N/P ratio, the luciferase activity was measured 24 and 48 hour post transfection

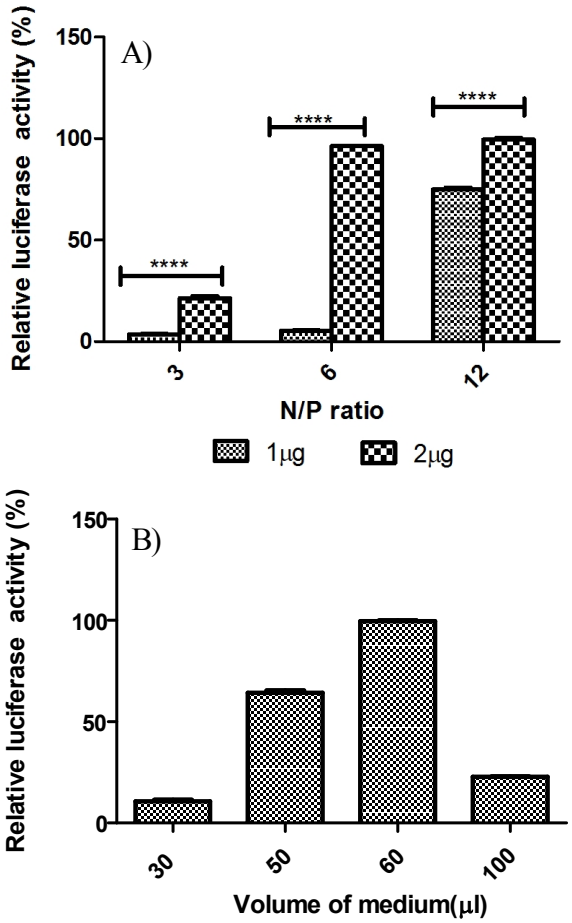

Fig. 5. The effect of Nanoparticles concentrations and medium on the cell during transfection efficiency. A) The MCF7 cells were transfected with nanoparticles contained different amount DNA at various N/P ratios. B) The MCF7 cells were seeded in 24 well plates and transfected with nanoparticles at N/P ratio 12, which was diluted at different volumes of DMEM medium and overlaid on cells for 4h

with the cell membrane and the initiation of endocytosis phenomena[19].

The effect of physicochemical properties of nanoparticles on transfection efficiency

Encouraging by suitable physicochemical properties, the transfection efficiency of NPs was also investigated in the different cell line. As shown in Fig. 4 the PEI/*psiCHECK* nanoparticle transfected BT474 cells in an N/P ratio dependent manner. In other words with increase of N/P ratio, transfection efficiency significantly increased. These results indicate a direct relationship between PEI content of nanoparticles and luciferase activity. These result may be due to increasing of the particles' charge which facilitated the cellular uptake and increase of PEI content of NPs also intensified endosomal escape of particles which in turn enhanced transfection efficiency [18-20]. The positive charge of nanoparticles also facilitated the interaction of nanoparticles with negatively charged components of the cell membrane; moreover, at N/P ratio less than 12 the nanoparticles showed lower size, which synergically promoted uptake of nanoparticles. Interestingly PEI/*psiCHECK* nanoparticles demonstrated maximum transfection at N/P ratio of 12 at 24h and 48 h similarly. At N/P ratio above 12,

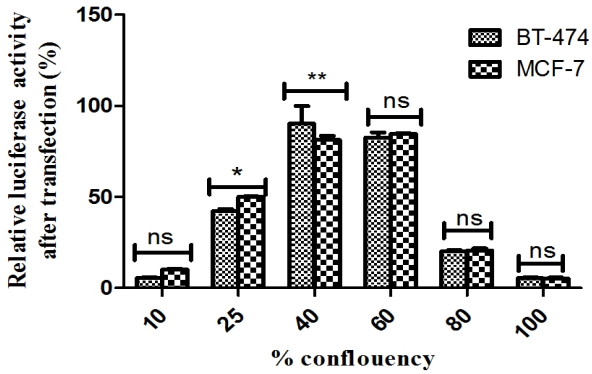

Fig. 6. The effect of confluency of cells on transfection efficiency in different N/P ratios

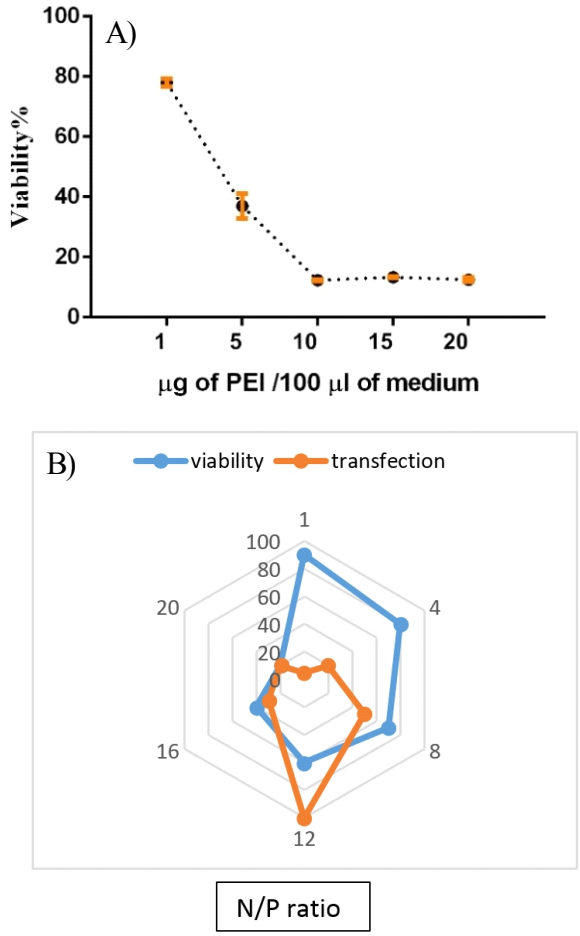

Fig. 7. Analysis of PEI cytotoxicity in MCF7 cell line and its relationship with transfection activity. A) Viability of cells in different amount of PEI. B) Viability of MCF-7 cells in differnet N/P ratios of nanoparticles and its relationship with transfection effeciency

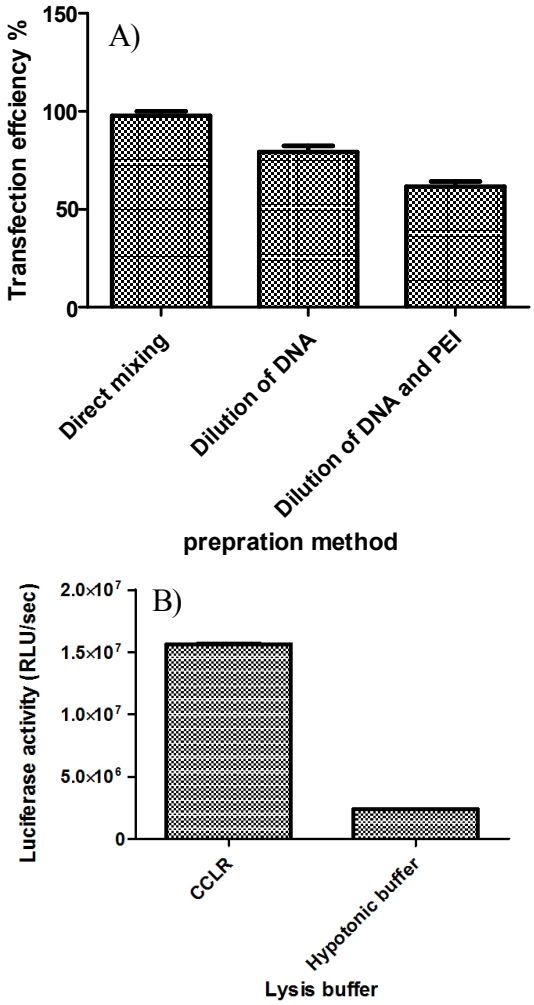

Fig. 8. Nanoparticle preparation method and its effect on transfection efficiency. a) PEI/*psiCHEK* Nanoparticles were prepared with different method at N/P ratio of 12 was transfected and luciferase activity was measured 48 h later. b) The HEK293T cells were transfected with PEI/*psiCHEK* Nanoparticles at N/P ratio of 12 and then 48 h later the cells were lysed by different agent

despite higher buffering capacity, unexpectedly the transfection efficiency was dramatically decreased. This result may be due to extra condensing of DNA, so prevented the release of the *psiCHECK* plasmid into the nuclear environment of cells, which were consistent with a lower intensity of ethidium bromide emitted from particles at higher N/P ratio. On the other hand, particle aggregation at high N/P ratio prevents from cellular uptake [21, 22].

The effect of nanoparticle concentration on the transfection efficiency

One of the main factors that control the cellular uptake of nanoparticles is their initial concentration. There are two approaches for control nanoparticle concentration; utilizing the different amount of nanoparticle at constant volume of cell culture medium and dilution of the constant amount of nanoparticles in various volumes of cell culture medium. The result was shown that increase of nanoparticle concentration with the both approaches, intensified the transfection efficiency (Fig. 5). For example, NPs prepared with 2µg of the psiCHECK plasmid at N/P ratio of 12 showed significantly more transfection efficiency compared to NPs at same N/P ratio, which prepared by 1µg plasmid. These results confirm a direct relationship between nanoparticle concentration and luciferase activity, which may be due to more uptake rate of nanoparticles. Alternatively, the decrease of the medium on the surface of cells during initial time of transfection increased the concentration of nanoparticles, which in turn increased transfection efficiency.

The effect of cellular confluency on the transfection rate

The confluency and growth phase of cells can play an important role in cellular response to the external particle. In deed growth phase of cells, which indirectly determined by confluency can ascertain the total transfection efficiency. In another effort, we have tried to know the optimum confluency of cell culture for transfection. For this purpose, we have prepared cell culture with different cell number per area and transfected with PEI/*psiCHECK* nanoparticles at N/P ratio of 12. The result demonstrated a significant relationship between cellular confluency and transfection rate. The increase in cell number has increased the transfection of the luciferase gene (Fig. 6). However, the luciferase activity drastically decreased at high confluency. This observation is probably, due to decrease of cell division due to density-dependent inhibition of cell division [23] and the presence of the nuclear envelope. It was shown that viral vector can enter nuclear envelope[24]. However, PEI/DNA nanoparticles due to lack of a specific nuclear delivery element failed to enter the nucleus of non-dividing cells. Moreover, contact inhibition at high confluency inhibits uptake of foreign DNA [25]. These results propose that confluency about 40-60% is suitable for transfection by PEI nanoparticles.

The cytotoxicity and its effect on transfection efficiency

Cytotoxicity is one of the common problems of synthetic carriers. Indeed non-biodegradable materials due to undesirable interact with cell component are mainly caused serious toxicity. In this study, we have examined the cytotoxicity of PEI/*psiCHECK* nanoparticles at various N/P ratios. As shown in Fig. 7, increase of NPs N/P ratio led to significant cytotoxicity. Moreover, the bare PEI at the same concentration, which was used in complex, caused more cytotoxicity. This high toxicity of bare PEI compared to the PEI/DNA complex, indicating that the high positive charge of free PEI might be one of the main cause of cytotoxicity. According to DLS result at high N/P ratio, nanoparticles showed more positively charges, which in turn caused more transfection and less cell viability. Therefore, N/P ratio of 12 could establish a balance between transfection and cytotoxicity, So that at this molar nanoparticles showed a suitable transfection meanwhile did not cause a significant cytotoxicity.

The effect of nanoparticle contact time with cell surface

In most of the transfection protocols, it was mentioned to exchange the medium after transfection. Although some were mentioned that PEI-based transfections, there is generally no need to change the medium after transfection[19]. In this study, we tried to know the effect of cell-nanoparticle contact time on the transfection rate. Therefore, NPs were either removed after 4 h or remained in the cell for 48 h. The result was shown that remain of nanoparticles more than 4 hours, cause to drastically increase of luciferase activity. This result suggested that nanoparticles are able to remain stable in cell culture and transfect new daughter's cells.

Optimization of nanoparticle preparation method

It has been shown that nanoparticles preparation method can affect the size and distribution of nanoparticles and thereby affect the transfection efficiency. In this study, we have examined three approaches for nanoparticles preparation: direct mixing of DNA with PEI, the addition of PEI to diluted DNA, mix of diluted DNA with diluted PEI. As shown in

Fig. 8a the nanoparticles which were prepared without dilution of its component showed higher transfection compare to other groups, presumably due to the production of the suitable complex for better uptake and release of DNA [26]. It should be determined in the next studies.

The effect of lysis buffer on the luciferase activity

One of the most important steps for analysis of nanoparticles for gene delivery is the development of suitable lysis buffer for enzyme assay. For this purpose, we have used CCLR and hypotonic buffers. The result was shown that CCLR buffer was better than the hypotonic buffer for display luciferase activity (Fig. 8b). This result may be due to the presence of triton x100 reagent in CCLR, which was suitable for luciferase enzymatic stability and better disruption of the cell membrane.

CONCLUSION

In conclusion, in the present study, we have evaluated the effect of physicochemical properties of PEI/pDNA NPs and cellular confluency on the transfection efficiency. The result demonstrates that control of size and charge of NPs could establish a balance between delivery rate and cytotoxicity. Moreover, at the end of growth stage can achieve to optimal transfection efficiency. Altogether, this study provides useful information for transfection of various nucleic acid by PEI based nanoparticles. This work demonstrates that N/P ratio is the main factor, which can affect the balance between the transfection and cytotoxicity of the nanoparticle. Moreover, transfection at logarithmic growth phase provides suitable transfection efficiency.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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