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RESEARCH NOTE

THE FORMULATION OF TRANSFECTION REAGENT FOR CHINESE HAMSTER OVARIES CELL CULTURE

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One of the most attractive concepts of biomedical applications is Gene therapy. Although the majority of previous gene delivery approaches involved adenoviral or retroviral vectors, non-viral approach is now receiving increased attention due to several advantages, such as its ease of manipulation, stability of cell, low cost procedure, biohazard and safety clearance, and high flexibility as gene delivery vehicles with regard to the size of the delivered transgene (Lollo *et al.,* 2000).

Various effective non-viral gene delivery approaches including naked DNA injection, physical techniques such as electroporation or gene gun and synthetic transfection reagents including cationic lipids (lipoplexes) and cationic polymers (polyplexes) have been developed (Godbey *et al.,* 1999). Non-viral synthetic vectors are essentially based on the condensation of negatively charged DNA into compact particles by electrostatic interactions with condensing compounds, protecting the DNA from degradation. They can also facilitate uptake into the cells via electrostatic interactions with anionic cell surface groups, such as proteoglycans. For sufficient transfection, a multistep process has to be mediated by the delivery vector which includes DNA condensation, uptake into the cell, endosomal release, migration through the cytoplasm, uptake into the nucleus, and finally de-condensation of the DNA into a transcribed form.

It has been revealed that Poly (ethylene-imine) is the most effective non-viral vector based on favorable characteristics of DNA protection, cell binding and uptake, endosomal escape and release from the carrier (Zhang *et al*., 2004). PEI of a certain high molecular weight is necessary for efficient delivery of DNA, however, a high molecular weight PEI is cytotoxic, and its long-term safety is problematic because of its non-biodegradability (Kircheis *et al*., 2001). Thus, to increase the transfection efficiency of PEI-based polyplexes and to reduce its cytotoxicity, various strategies have been formulated. Several groups have reported the potential cytotoxicity against various cell lines of high molecular weight PEIs such as PEI (Mw = 25k) (He *et al.*, 2010). Also, the high affinity of PEI (Mw = 25k) for DNA is another important barrier to cytosolic delivery which would limit the overall transfection efficiency due to the relatively inefficient dissociation of pDNA from PEI. On the other hand, low molecular weight (LMW) PEI is less toxic but shows almost no transfection (Godbey *et al*., 1999). Therefore, modification of low molecular PEIs has been studied extensively to improve gene transfer efficiency while keeping cytotoxicity manageable (Choi and Lee, 2008).

While effective non-viral delivery systems try to imitate the effective mechanisms for DNA packaging, cellular uptake and nuclear uptake of viral vectors, non-viral vectors have also introduced new mechanisms unknown to viruses, such as the 'proton sponge' mechanism of PEI for endosomal release. Between the various synthetic vectors, polyethylenimines (PEIs) have shown particularly promising efficiency in transfections in cell culture as well as in a variety of applications *in-vivo*. As several intrinsic features necessary for efficient transfection, such as DNA condensation and endosomal release are already intrinsic to the PEI molecule, additional modifications have been introduced to add target specificity and improve the biocompatibility for *in-vivo* application. The transfer of genetic material through an appropriate vector, which can be a viral or nonviral vector, into the target tissue is termed an *in vivo* delivery. This technique is the least advanced strategy at present but potentially, it might be the most useful.

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Although the problem of this approach is insufficient targeting of vectors to the correct tissue sites, improvement in targeting and vector development present opportunities to solve the problem (Nouri *et al.,* 2012).

The aim our present study is thus to explore the effect of different concentrations of polyethylenimines (PEIs) on transfection efficiency in Chinese Hamster Ovaries cell culture.

Transformed *E. coli* DH5α cells with plasmid EGFP was plated on the LB agar containing 30μg/mL kanamycin. The cloned plasmid DNA was subsequently isolated using a plasmid isolation kit (PureLinkTM Quick Plasmid Miniprep, Invitrogen TM). Using this kit, cells were lysed using an alkaline procedure and plasmid to be transfected into the CHO cell was purified from the strain.

CHO (Chinese Hamster Ovary) cell line was supplied by University of Leeds, United Kingdom (American Type Culture Collection). Mammalian cell line, Chinese Hamster Ovary (CHO) cells, was maintained as monolayer cultured in Dulbecco's modified Eagles (DMEM, SIGMA) supplemented with 10% fetal bovine serum (FBS), 2%L- Glutamine and 1% antibiotics(all were from Invitrogen, UK).

Using aseptic techniques, plastic vial and six-well plates were used as culture flasks to subculture the CHO cells. Culture vial medium that contain CHO cells were discarded and cells were washed several times with 2 ml PBS to remove traces of serum that could inhibit the reaction of trypsin. Trypsin was added into the culture vial and left stationary for about 1 minute for the detachment of cells from the vial. Trypsin was thereafter removed from monolayer (few drops were retained). New complete medium was then added into the culture vial and the cells were dispersed by repeated pipetting of the medium over the surface of the monolayer. These was subsequenty incubated at 37°C and 5% CO_2 atmosphere in humidified incubator until the 70–80% confluency was reached (Lampela *et al.,* 2002). This process was repeated after 2 to 3 days to maintain the viability of the mammalian cells.

Branched PEI25K and linear EI22K were obtained as transfection reagents (Sigma–Aldrich, USA). PEI and EI stock solutions at a concentration of 200 mg/ml were prepared using distilled water which had been heated to 80°C. The solutions were then allowed to cool to room temperature before adjusting the pH to 7.0 with 5M HCl, filter-sterilized and stored at -80°C.

Solution A (plasmid DNA diluted in Serum Free Medium) was prepared 5 minutes prior to mixing with Solution B (fuGENE6 reagent diluted in serum free medium). After mixing, the solution mixture was incubated for 15-45 minutes at room temperature to allow PEI/DNA complex formation before being added to the Chinese Hamster Ovary cell culture which was previously washed with PBS. The cell culture mixture was incubated for another 5 hours in serum free medium. Then, the cells were washed and sub-cultured in fresh complete medium for 24 hours, and were analyzed using immunofluorescence microscope at 10x magnification to successfully identify the transfected cells. FuGENE6 reagent without plasmid was used as a positive control.

Four hours before transfection, the CHO cells were washed with PBS and replaced with fresh media containing 10% FCS. Plasmid DNA sample (diluted in 50 μl basal medium without additives, serum, antibiotic or other proteins) was mixed with various concentration of PEI or EI reagent (0.2-200 mg/ml for separate EI and PEI reagents and 0.2-2 mg/ml for PEI/EI mixture). These were incubated for 8 minutes at room temperature before being added to the CHO cells. After the addition of reagent mixture, the CHO cells were incubated at room temperature for 40 minutes, followed by further incubation for 24 hours at 37°C incubator before being analyzed using immunofluorescence microscope at 10X magnification to identify successfully transfected cells.

Transfected cells were viewed using an inverted fluorescence microscope under 10X objective lens to identify the successful transfected cells. All experiments were run in triplicates and analyzed with SPSS software using one way ANOVA. Results were considered to be statistically significant at $p<0.05$.

DNA must reach the nucleus of the cell and become accessible to the transcription machinery. The main aim of transfection was to thus allow the DNA to reach the nucleus of the cell and become accessible to the transcription machinery. Each experiment was begun by seeding approximately $5x10^5$–$1x10^6$ cells into 6 well plates with total medium volume of 5ml per well. The cells were incubated for 4 hours before transfection.

The numbers of transfected cells were counted after 24 hours of incubation and green fluorescence showed the expression of the transfected cell reporter gene (Fig. 1). High concentration of plasmid results in high transfection efficiency in this study. Many parameters such as molecular weight and type of polycationic polymer can also affect the level of protein expression.

In this study, different concentrations of PEI and EI reagent were used as transfection reagent to determine the optimum concentration of PEI and EI reagent with the highest transfection efficiency. Results showed that there were many dead cells in both PEI and EI reagents. Changes in morphology of the cells were observed as the cells showed abnormal shapes and clumping.

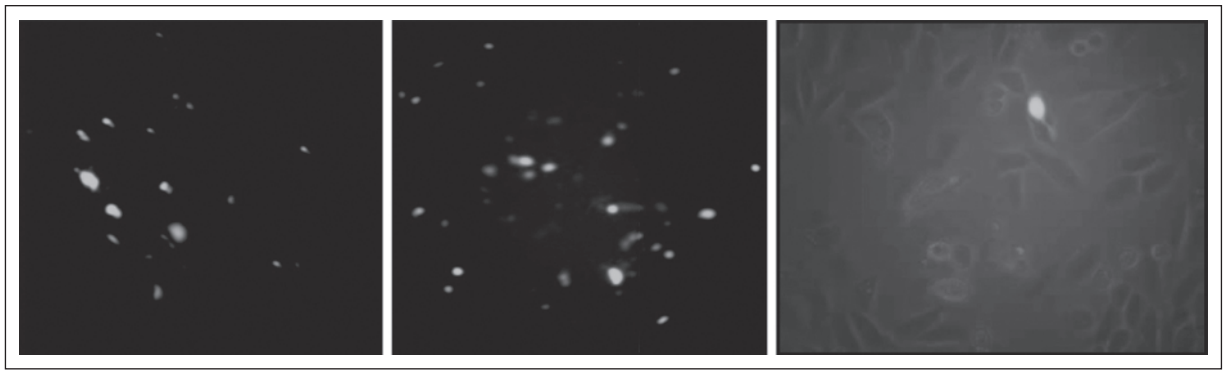

Fig. 1. Comparison of transfection efficiency of various concentrations of PEI-DNA complexes. CHO cells were incubated with PEI-DNA complexes and incubated for 24h. The Green Florescent Protein (GFP) expression was observed under fluorescent microscope at 10x magnification.

As shown in Fig. 2a and b, there were significant ($p<0.05$) number of transfected cells observed in transfection experiment using 1.8 mg/ml of PEI reagent, while 1.2 mg/ml of EI reagent resulted in the highest number of transfected cells as compared with other concentrations. When compared with positive control, all concentrations of PEI reagent showed significantly lower percentage of transfected cells except the concentration of 1.8 mg/ml. As for EI reagent, there were no significant changes in the percentage of transfected cells as compared to positive and negative controls. However, EI reagent at a concentration of 1.2 mg/ml showed slightly higher percentage of transfected cells as compared to the positive control.

PEI was shown to have higher transfection efficiency when packed with reporter plasmid EGFP as compared to EI. This might be due to the greater buffering capacity of branched polycationic polymer (PEI) as compared to linear polycationic polymer (EI). However, PEI possesses toxic effect on CHO cells because of the permeabilizing action of PEI on plasma membrane of mammalian cells. Besides, the positive charge of the polycationic polymer also plays a pivotal role in cellular toxicity of the cells. Polycationic polymer forms strong electrostatic interaction with proteins on the plasma membrane of these cells leading to destabilization and rupture of the cell membrane structure.

FuGENE6 was used as positive control in the ratio of 3:1 (Fugene6:DNA) (μg/μl). Mixture of CHO cells and plasmid DNA sample without transfection reagent was used as negative control. Values were expressed as mean ± S.E.M. with triplicates in each group. Results were compared with positive and negative controls. Values are statistically significant at $^{a}P<0.05$ as compared to negative control and $^{b}P<0.05$ as compared to positive control.

In order to determine the effects of different concentrations of PEI/EI mixture reagent on transfection efficiency, constant amount of plasmid DNA and PEI/EI (mix polycationic polymer) reagent was held at a ratio of 1:1 (μg/μg) (Fig. 3). As shown in Fig. 3, the optimal concentration of PEI/EI mixture which produced the highest transfection efficiency was 1.2 mg/ml. In addition, CHO cells transfected with plasmid DNA using 1.2 mg/ml of PEI/EI mixture transfection reagent were observed to show normal morphology.

FuGENE6 was used as positive control in the ratio of 3:1 (FuGENE6: DNA) (μg/μl). Mixture of CHO cells and plasmid DNA sample without transfection reagent was used as negative control. Values were expressed as mean ± S.E.M. with triplicates in each group. Results were compared with positive and negative controls.

Morphology of the CHO cells were examined under inverted fluorescence microscope 48 hours after transfection with different concentrations of polycationic reagent (Fig. 4). Based on the results, many of the cells were dead with abnormal shape and formed huge clumps. This might be due to over-expression of the EGFP plasmid, toxicity effect of the polycationic reagent on the cells or confluency of the cells caused by inappropriate technique that has been practiced while preparing the sample. The appropriate confluency of the CHO cell to be transfected must be around 80 to 90 percents confluency because if the density of cell is too high, inhabitation of cell growth could occur. Conversely if cell density is too low, recovery of the culture from transfection can be low.

In this study, plasmid containing pEGFP was transfected into CHO cells. The efficiency of transfection in different concentrations of PEI and EI reagents were studied and compared with FuGENE6 commercial transfection reagent. Results showed that the optimum concentration of PEI reagent for efficient transfection process was 1.8 mg/ml. Meanwhile the optimum concentration of EI and PEI/EI mixture reagents were 1.2 mg/ml, respectively. Therefore, PEI and EI reagents in the

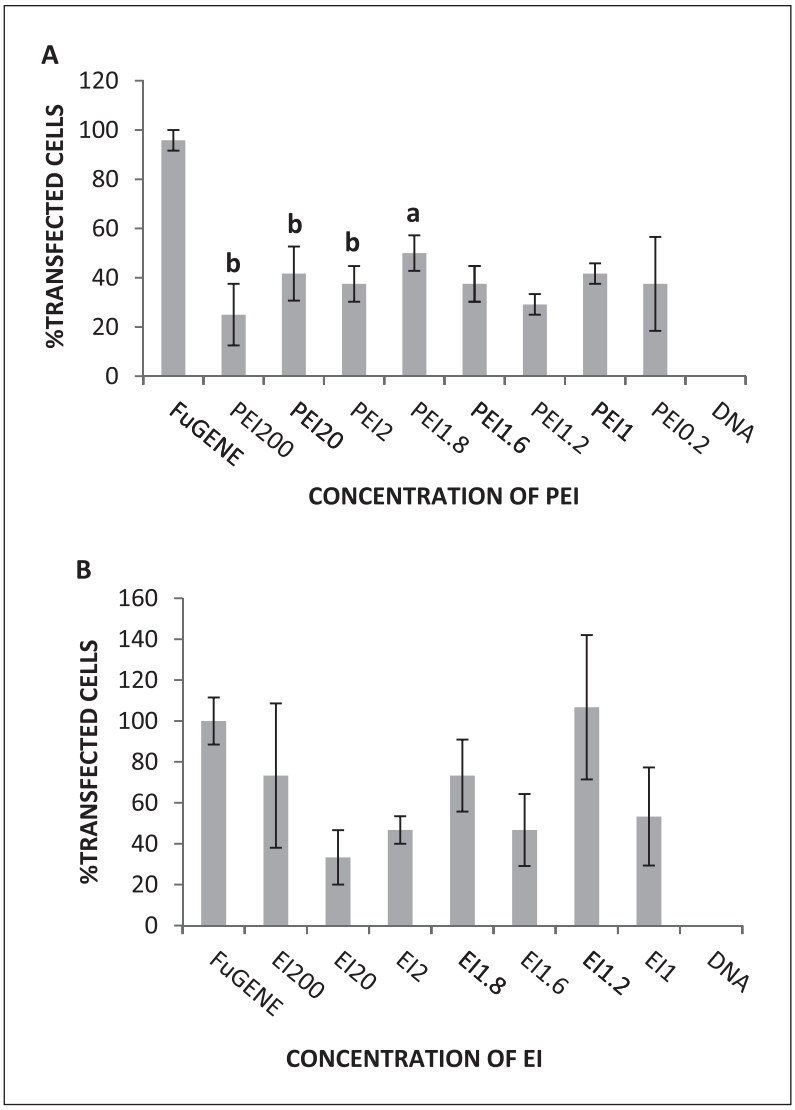

Fig. 2. Effects of different concentrations of PEI (A) and EI (B) in a constant amount of PEI: DNA 1:1 (μg/μg) and EI:DNA 1:1 (μg/μg).

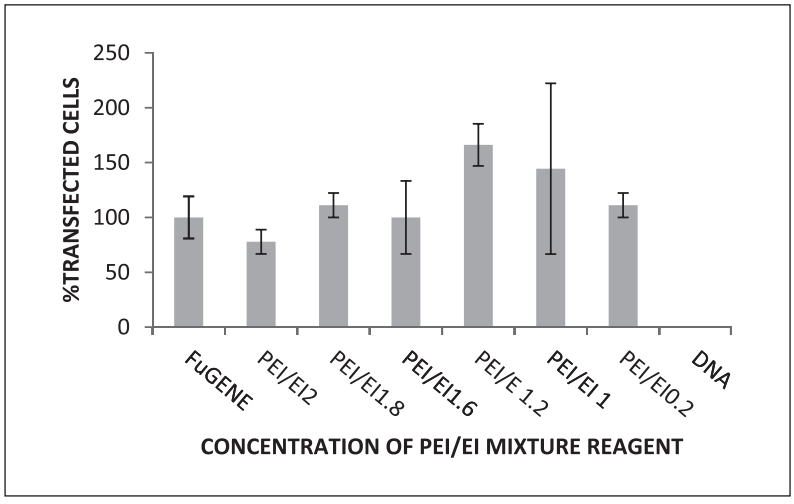

Fig. 3. Effects of different concentrations of the (PEI/EI) reagents in constant amount of the PEI/EI reagent mixture: DNA (1:1) (μg/μg).

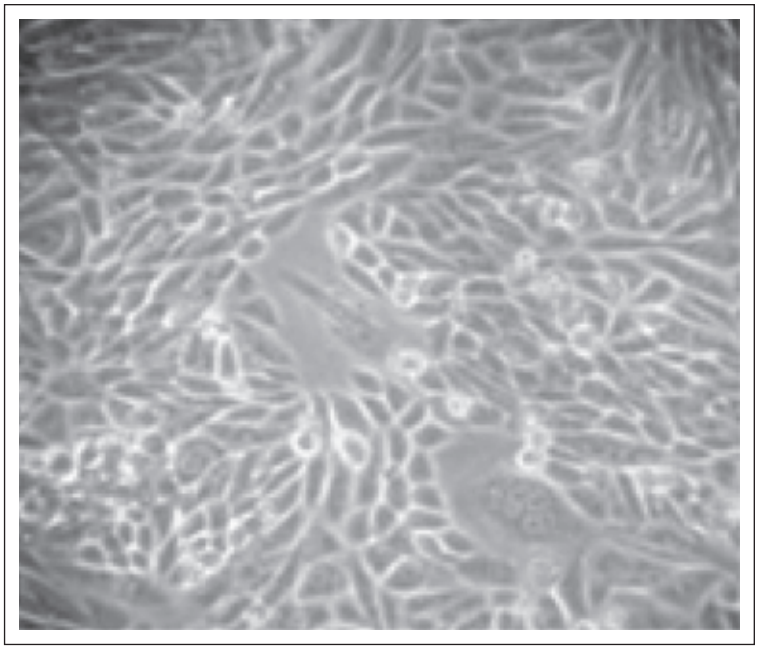

Fig. 4. Morphology of CHO cells after 48 h of transfection in different concentrations of (PEI).

ratio of 1:1 with plasmid DNA could be used as an efficient transfection reagent with the concentrations determined in this study.

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