Preparation of a Low Molecular Weight Polyethylenimine for Efficient Cell Transfection

BioTechniques 30:74-81 (January 2001)

ABSTRACT

Polyethylenimines (PEIs) of a molecular weight between 25 and about 800 kDa have successfully been used for in vitro and in vivo gene delivery approaches. Recent publications indicated that PEI molecules of lower molecular weight and a small molecular weight range are also efficient transfection reagents with a much lower cytotoxicity compared to high molecular weight PEIs. Here, we describe the application of a molecular sieve chromatography to fractionate a commercially available 25-kDa PEI. We generated three pools of PEIs with molecular weight ranges of 70–360 (I), 10–70 (II), and 0.5–10 kDa (III), respectively. We show that, in comparison with the 25-kDa PEI, pool III increased the expression of luciferase up to 100-fold and the number of transfected cells 2–3-fold. In addition, the kinetics of reporter gene expression was also much faster in pool III, compared with the 25-kDa PEI or with pools I or II. Finally, pool III showed the lowest cytotoxicity in comparison with the other PEI preparations. Thus, we provide a one-step processing of a 25-kDa PEI, resulting in a more effective and also less cytotoxic transfection reagent.

INTRODUCTION

Polyethylenimines (PEIs) are cationic polymers that are commercially available in a broad molecular weight range (200–800 000 Da) and with different degrees of branching (5,18). Although the different PEI preparations are labeled with a certain molecular weight, most preparations consist of PEI molecules with a broad molecular weight distribution (12,18). Because of the high number of amino groups, PEIs carry a strong positive surface charge, which make them suitable to bind and package large negatively charged molecules such as DNA (6,9). For this reason, PEIs have successfully been used for in vitro and in vivo gene delivery experiments with higher eukaryotic cells (1,2,4,5,11,16). Originally, it was thought that PEIs of higher molecular weight were superior over low molecular weight PEIs in their transfection efficiency (12). However, PEIs of higher molecular weight are cytotoxic and can be used in transfection experiments only in a narrow concentration range and for a restricted incubation time (11).

In a recent paper (11), we described the synthesis of a PEI of low molecular weight (average M_r = 10 000 Da), a narrow molecular weight distribution (8000–12 000 Da), and a low degree of branching. We could show that this low molecular weight PEI can successfully be used for gene delivery in vitro, avoiding the cytotoxic effects that are induced by higher molecular weight PEIs. Here, we describe an easy method to fractionate a 25-kDa PEI by standard molecular sieve chromatography. Using different fractions for transfection experiments, we observed an up to 100-fold increase of reporter gene expression with a decrease in molecular weight.

MATERIAL AND METHODS

Molecular Sieve Chromatography

As a starting material, we used the PEI preparation from Sigma (St. Louis, MO, USA) with an average molecular weight of 25 000 Da (Al 25-kDa). This PEI was further fractionated in a molecular sieve chromatography column of 1.5 cm in diameter (Bio-Rad Laboratories, Hercules, CA, USA) and 50 cm in length. To pack the column, 3 g Sephadex® G-50 fine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were incubated in 300 mL distilled water for 1 h at 4°C. Then, 200 mg PEI were added, and the mixture was incubated overnight at 4°C to block possible binding sites for PEI on the Sephadex beads. The column was packed with a gel bed 40 cm in length and equilibrated with water under a flow of 1.2 mL/min obtained by a minipuls 2 pump (Abimed, Langenfeld, Germany). Samples were dissolved in deionized water and applied in 1-mL volumes. PEIs of different molecular weight (0.8, 2, 800; all from Sigma; and 10 kDa PEI; see Reference 11) were used as molecular weight standards. PEIs were applied in a concentration of 50 mg/mL. Fractions (790 µL) were collected, and the concentration of PEI in each fraction was measured as described below.

Quantification of PEI

One hundred microliters of each fraction were added to 900 µL 0.02 M cupric acetate in 5% potassium acetate, pH 5.5. The probes were mixed well, and absorption was measured at 690 nm using a spectral photometer (Hitachi, Tokyo, Japan). The PEI-containing fractions were pooled as indicated, and the PEI concentration of each pool was calculated from a calibration curve that had been obtained with 0.5–250 µg PEI.

Transfection of Cells

For gene transfer studies, the pancreatic carcinoma cell line PaTu 8902 (10) was used. Cells were seeded with 2.4×10^4 cells/cm^2 and were grown as adherent culture in DMEM supplemented with 10% fetal calf serum, 10% horse serum, 2 g/L HEPES, gentamycin at 37°C, and 5% CO_2 in a humidified chamber. Twenty-four hours after seeding, the medium was changed to a volume of 2.5 mL. As transfection reagent, either the original 25 000 Da PEI or fractions of this PEI obtained by molecular sieve chromatography (see above) were used. The pCMV sGFP LucIAV expression vector used (Reference 7; plasmid was kindly provided by Richard Day, Health Science Center, Charlottesville) encoded a dual-function fluorescent and luminescent reporter protein driven by the cytomegalovirus (CMV) promoter. Plasmid was grown in XL1 competent *E. coli* cells under 50 mg/L ampicillin and was isolated using a maxiprep kit (Genomed, Raleigh, NC, USA). Per condition, three 21-cm^2 dishes were used. To transfect cells, PEI/DNA complexes with a N:P ratio of 67 were used. Plasmid DNA (10 µg) and 100 µL PEI stock solution (0.9 mg/mL, pH 7.4, sterile filtered) were seperately diluted into 750 µL each with 150 mM sodium chloride. After 10 min at room temperature, the PEI solution was pipetted

dropwise to the DNA. After 10 min more at room temperature, the PEI-DNA solution was mixed, and 500 µL were added to 2.5 mL culture medium. Cells were incubated under conditions as described above for 6 h. The medium was changed again, and cells were further incubated for 40 h.

Measurement of Luciferase

Cells were washed with neutral PBS and scraped off the dishes in 400 µL lysis buffer provided with the luciferase detection Kit (Promega, Madison, WI, USA). After two freeze and thaw cycles, the lysate was centrifuged at 13 000× *g*, and 10 µL supernatant were used together with 100 µL substrate solution to measure relative light units (RLUs) in a scintillation counter PW 4700 (Philips, Eindhoven, The Netherlands) equipped with a program to measure luciferase activity. To adjust the RLUs to the amount of cells, the relative amount of DNA was measured using a Hoechst 33258 dye stock solution (1 mg/mL 2 M sodium chloride and 25 mM Tris-HCl, pH 7.5; Polysciences, Warrington, PA, USA) in a

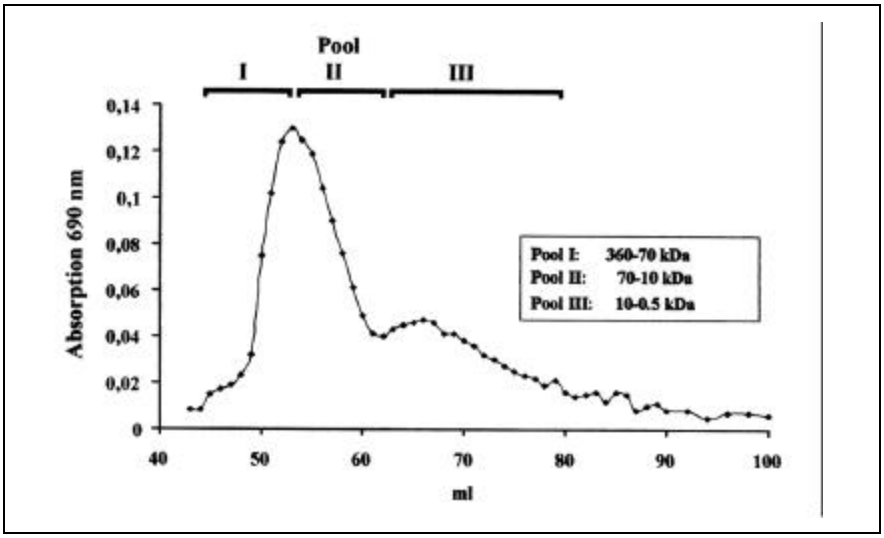

Figure 1. Molecular sieve chromatogram of Al 25-kDa PEI using a Sephadex G-50 molecular sieve. Fractions were pooled as indicated, and molecular weight ranges determined using PEIs of different sizes.

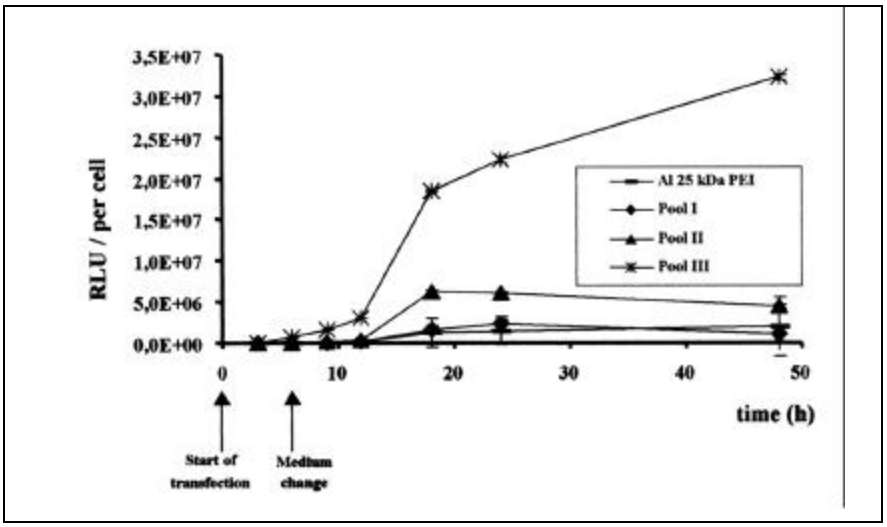

Figure 2. Kinetics of luciferase expression after transfection of PaTu 8902 cells with the Al 25-kDa PEI and the different PEI pools described in Figure 1. PaTu 8902 cells were transfected and harvested at the time points indicated.

1:1000 dilution. Fluorescence was measured using a fluorescence spectrophotometer at an excitation wavelength of 365 nm, an emission wavelength of 460 nm, and a high voltage of 450 V. Data are expressed either as RLU per dish or RLU per Hoechst fluorescence intensity. Standard experiments included three individual dishes per experimental condition, together with two further dishes, which had received 500 µL 150 mM sodium chloride only during the transfection procedure. The mean values of RLUs from these control cells were subtracted from the RLUs of the transfected cells as background, which was between 5000 and 10 000 RLUs.

Fluorescence Microscopy

Cells transfected with pEGFP as a reporter gene were quantitated by fluorescence microscopy using an Axiovert inverted microscope (Carl Zeiss, Jena, Germany) equipped with an excitation band pass filter (450–490 nm), an emission long pass filter of 520 nm, and a dichroic beam splitter of 510 nm. For quantification of all cells, nuclei were counterstained with the Hoechst 33258 dye. To visualize the Hoechst fluorescence, we used an excitation band pass filter (365–375 nm), an emission long pass filter (397 nm), and a dichroic beam splitter of 395 nm. To obtain the percentage of green fluorescent protein (GFP)-positive cells, eight independent areas per condition were calculated for GFP-positive and Hoechst dye-positive cells, respectively. The total amount of cells counted per condition was 2000–2800.

Lactate Dehydrogenase (LDH) Assay (19)

PaTu 8902 cells were transfected for 6 or 24 h, as described above, using either the 25-kDa PEI or pools obtained from the 25-kDa PEI by molecular sieve chromatography. After incubation, cells were washed twice with PBS, and 400 µL lysis buffer (Promega) per dish were added. After two freeze-thawing cycles, cells were harvested, and 50 µL were mixed with 1 mL reaction solution containing 56 mM Tris-HCl, pH 7.4, 5.6 mM EDTA, 0.45 mM sodium pyruvate, and 282 µM NADH. Changes in absorbance were read at room temperature for 3 min at 340 nm, and LDH activity was calculated from the slope of the linear curve obtained (19).

RESULTS AND DISCUSSION

PEIs have been successfully used for gene delivery under in vitro and in vivo conditions (1,4,5,15). This cationic polymer is commercially available in a variety of preparations that differ in size and in the degree of branching. Previous publications using PEIs of different molecular weight came to the conclusion that transfection efficiency increased with increasing size of the PEI molecules (12). However, high molecular weight PEIs also have a high cytotoxicity, limiting the concentration range in which PEIs can be used and the duration of incubation (11). Recently, we described the synthesis and use of a 10-kDa PEI, which combined a high transfection efficiency with an extremely low cytotoxicity (11). This led us to

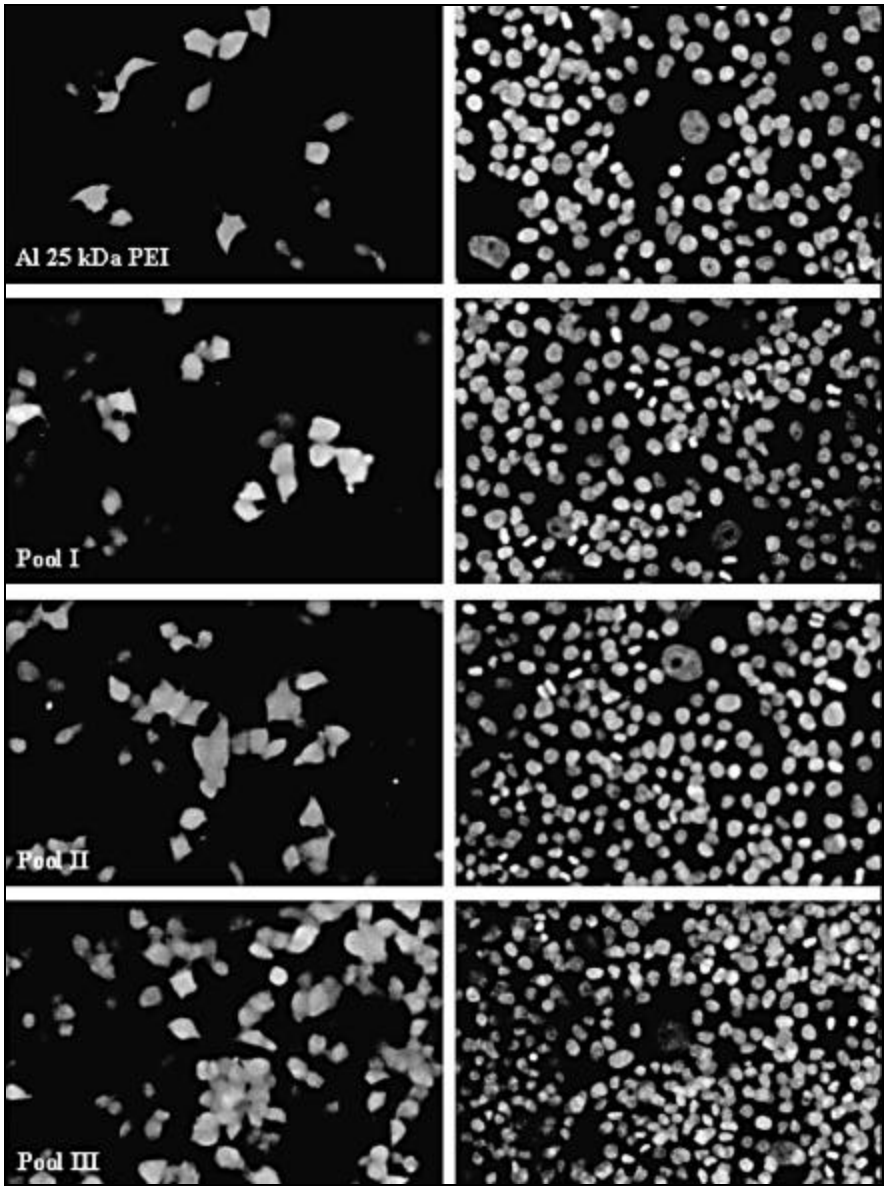

Figure 3. PaTu 8902 cells transfected with different PEI preparations, using the GFP as reporter gene (left panel). Cells were fixed 24 h after transfection and counterstained with Hoechst 33258 (right panel). For quantitative data, see Materials and Methods.

our approach of subfractionating the commercially available 25-kDa PEI used in this study to obtain PEI pools of lower molecular weight. For fractionation of Al 25-kDa PEI, we applied a molecular sieve chromatography using Sephadex G-50, as described in Material and Methods. From the chromatogram shown in Figure 1, PEI-containing fractions were assigned to three pools as indicated (I, fractions 45–53; II, fractions 54–62; III, fractions 63–79). Different PEIs were used as molecular weight standards (see Materials and Methods), and the molecular weight range of each pool was calculated (I, 70–360 kDa; II, 10–70 kDa; III, 0.5–10 kDa). This analysis revealed that the 25-kDa PEI used has a broad molecular weight range of 0.5–360 kDa. PEI from pool III is comparable with the 10-kDa PEI mentioned above, which had a molecular weight range of 8–15 kDa (11).

To compare the expression levels of the different pools with the original Al 25-kDa PEI, we used luciferase as reporter gene driven by a simian virus 40 (SV40) promoter. Significant differences between AL 25-kDa PEI and the pools were observed. Depending on the molecular weight of the PEI used, the RLUs measured 48 h after the start of transfection and corrected for the amount of cells were 1.9×10^6 RLUs for Al 25-kDa PEI, 1.1×10^6 RLUs for pool I, 4.4×10^6 RLUs for pool II, and 3.2×10^7 RLUs for pool III (data from Figure 2, time point 48 h). Thus, the expression level of luciferase increased 17-fold between the Al 25-kDa PEI and pool III. In other experiments, there was an up to 100-fold increase of luciferase expression level between Al 25-kDa PEI and pool III at 48 h after transfection (data not shown). Interestingly, at least pool II and pool III led to a higher expression of the reporter gene than the original Al 25-kDa PEI, although the same amounts of polymer and the same P:N ratio were used. We suppose that the relative amount of small PEI aggregates, at least in pools II and III, is higher than in the Al 25-kDa PEI, enhancing transfection in a not yet understood mechanism.

When the time course of the luciferase expression was determined, Al 25-kDa PEI and pools I and II luciferase expression revealed comparable kinetics (Figure 2). Eighteen hours after transfection has been started, Al 25-kDa PEI and pools I and II reached their maximum of luciferase expression. Longer incubation times did not lead to a further increase of the luciferase activity. In contrast, pool III revealed an earlier onset of luciferase expression, and 48 h after the start of transfection, the luciferase activity per cell was still increasing. Our data indicate that the enrichment of small PEI molecules with a narrow molecular weight range enhances transfection efficiency and transfection kinetics markedly. In accordance with data published earlier (11), the differences in luciferase expression suggest a faster uptake and/or a faster intracellular processing of the PEI/DNA complexes when smaller PEI molecules are used.

To measure the transfection efficiency in terms of the number of cells transfected, GFP was used as the reporter gene. The results were compatible with those obtained with luciferase as the reporter gene; however, the increase between the 25-kDa PEI and pool III was

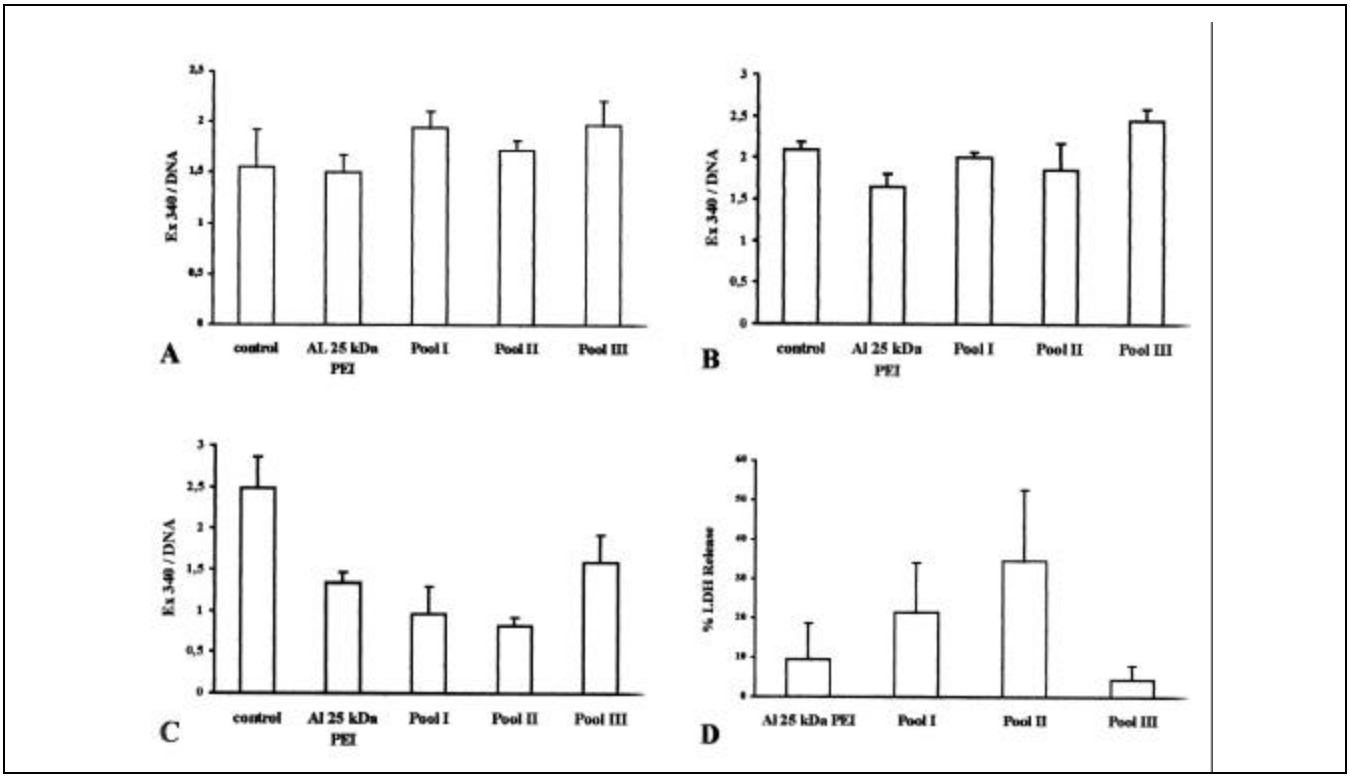

Figure 4. LDH release from cells treated with different PEI as indicated. Control, transfection without PEI. LDH activity was corrected for cell numbers using the Hoechst 33258 dye assay. LDH was measured 6 h (A and B) and 24 h (C and D) after transfection in the cell culture medium (A and C) and in the cells (B and D).

only 2–3-fold, instead of up to 100-fold as seen with luciferase as reporter gene (Figure 3). For Al 25-kDa PEI, 9.7% ± 4.5% of the PaTu 8902 cells were transfected. The percentage of transfected cells rose to 14.7% ± 2.9% with pool I, to 19.5% ± 2.2% with pool II, and to 26.4% ± 3.0% with pool III, respectively. This indicates that both the expression level per cell of transfected cells and the number of transfected cells are increased.

The enhancement of cell transfection with smaller PEI molecules can be based on a more efficient uptake of PEI/DNA complexes when low molecular weight PEI is used. Furthermore, the intracellular release of the PEI/DNA complex could be more efficient with low molecular weight PEI molecules. It is thought that PEI/DNA complexes enter the cell via an endocytotic pathway (4,13,20), where they are faced with an acidic pH. A popular hypothesis assumes that, under low pH, PEI molecules led to a rupture of the endosomal or lysosomal membrane so that the DNA/PEI complexes have access to the cytoplamic space (3,14,20). Smaller PEI molecules or molecule complexes could be packaged with higher density, promoting PEI/DNA complex release. Finally, the strength of the binding between DNA and PEI could be different, depending on the size of PEI molecules. PEI/DNA complexes are very stable molecular assemblies in which the access to the DNA is difficult. For example, in experiments in which bromodeoxyuridine (BrdU)-labeled DNA was used, an antibody against BrdU could not bind to the DNA when it was complexed with PEI (data not shown). The strong binding between PEI and DNA makes it unlikely that transcription can be initiated on a complexed DNA. Thus, a separation of PEI and DNA seems to be another critical step for the efficiency of transfection, and this step could be a function of the molecular weight of the PEI molecules used. However, so far, no data are available about the occurrence and the mechanism of the intracellular separation of PEI/DNA complexes.

To test whether the different pools and the original Al 25-kDa PEI induce cytotoxic effects, the intracellular amount of LDH was determined after a 6-h incubation period with the different PEI preparations (19). Nontransfected cells were used as a control. Under these conditions, no LDH activity was detectable in the culture medium. When LDH activity was measured in cell homogenates, no significant difference was observed between cells transfected with Al 25-kDa PEI, transfected with pools I-III, or nontransfected cells, indicating that none of the PEIs are cytotoxic under the experimental conditions used (Figure 4, A and B). Furthermore, no morphological alterations of the transfected cells could be detected (data not shown). However, cells incubated for 24 h with the different PEI/DNA complexes exhibited a decrease of cellular LDH activity (Figure 4C). Furthermore, LDH activity could now be detected in the culture medium (Figure 4D). LDH release, and thus cytotxicity, was lowest when pool III was used for transfection (4.3%), compared to Al 25-kDa (9.6%), pool I (21.4%) and pool II (34.7%).

The mechanism of the cytotoxic effect of PEI molecules is not understood. The most attractive hypothesis is that PEI aggregates on the cell surface in large clusters, which impair membrane functions and the membrane turnover, leading to cell necrosis (11,17). This aggregation of high molecular weight PEIs has been shown by electron microscopy, where clusters were observed with diameters larger than 2 µm (11). In contrast, in the case of a 10-kDa PEI, only small aggregates had been shown of a mean diameter of about 40 nm (11). Whether low molecular weight PEI molecules also alter membrane function or whether they develop a cytotoxic potential after they have been taken up by cells remains to be clarified.

In conclusion, we put forward a simple processing of a commercially available 25-kDa PEI to obtain a pool of PEI molecules with a low molecular weight and a small molecular weight range (0.5–10 kDa). This preparation has proved to be highly efficient in transfection experiments with up to a 100-fold increase in expression of the reporter gene luciferase and a 2–3-fold increase in the number of transfected cells using GFP as reporter gene. Under standard transfection conditions (incubation of cells with PEI/DNA complexes for 6 h), none of the PEI preparations used were cytotoxic in accordance to LDH release from the cells.

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This work was supported by the P.E. Kempkes Foundation, Marburg, and by the Dr. Hilmer Foundation, Essen. *Address correspondence to Dr. H.P. Elsässer, Department of Cell Biology, Robert-Koch Str. 5, 35033 Marburg, Germany. e-mail: elsaesse@mailer.uni-marburg.de*

Received 11 May 2000; accepted 28 August 2000.

Thorsten Bieber and Hans-Peter Elsässer
University of Marburg
Marburg, Germany

Acetylcholinesterase Assay for Rapid Expression Screening in Liquid and Solid Media

BioTechniques 30:81-86 (January 2001)

ABSTRACT

The synaptic enzyme acetylcholinesterase (AChE), which is the target of many insecticides and potential warfare agents, is implied in Alzheimer's disease and is a good potential candidate to be used in biosensors. This promotes a strong demand for production of recombinant AChE to be used in various studies. A promising expression system is the yeast Pichia pastoris*, but the expression efficiency needs to be improved. Optimization studies require a rapid and efficient screening test to detect positive yeast colonies after transformation. Using indoxylacetate as a substrate, we designed a chromogenic test that is not interfered with by the culture media background color and, thus, is suitable for microplate screening. Moreover, it was possible to adapt the test for direct on-plate detection of AChE-expressing colonies.*