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Transfection improvements of fish cell lines by using deacylated polyethylenimine of selected molecular weights

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ABSTRACT

A new tool for DNA transfer to fish cell lines such as *epithelioma papulosum cyprini* (EPC) and rainbow trout gonad (RTG2), has been optimized by testing commercially available polyethylenimine (PEI) polymers as transfectant reagents. Deacylated 25 kDa PEI polymers were selected amongst the most active and then low toxicity deacylated PEIs fractions around 15 kDa were obtained by gel filtration chromatography to increase 3–4-fold their initial *in vitro* transfection efficiency. The EPC and plasmids coding for reporter genes were first used to optimize variable values for best expression by transfection with deacylated low toxicity PEI while both EPC/RTG2 and a plasmid coding for the glycoprotein G gene of the fish pathogen, viral haemorrhagic septicemia virus (VHSV) were then used to demonstrate some of their practical applications. Due to its relatively low price, defined chemical composition and availability, low toxicity deacylated PEI might be used for numerous applications for all those studying fish cell immunology *in vitro* as well as *in vivo*.

1. Introduction

The *epithelioma papulosum cyprini* (EPC) from carp [1] and the rainbow trout gonad (RTG2) from trout [2] are amongst the most useful fish cell lines for fish immunology-related research. *In vitro* assays for expression and effects of cloned immune response-related fish genes [3–5] as well as for any heterologous gene expression in fish cells [6], immune or fusion assays with transfected fish rhabdoviral glycoprotein genes [7–9], possible improvement of fish DNA vaccination methods [10–14], search for new promoters active in fish cells [15,16], rescue of immunologically attenuated infectious RNA fish viruses from cDNA copies [17] or studies on siRNA interference on permanently transformed fish cell lines [18], are among the many examples which should benefit from improvements of *in vitro* DNA transfection of EPC and/or RTG2 fish cell lines. Furthermore, improvement of RTG2 transfection will help possible *in vitro* studies requiring MHC class I-matched effector and target fish cell systems, now that homozygous isogeneic rainbow trout clone C25 and the RTG2 cell line were shown to have identical single dominant polymorphic MHC class I locus Onmy-UBA*501 alleles [19,20].

Transfection of EPC [1] was first reported by using the calcium chloride method [21]. The efficiency of transfection was later improved by the use of commercially available liposomes such as Lipofectamine®, Fugene 6®, TransIT LT1®, and others, but their chemical composition is not available (unrevealed because of commercial reasons) [18,22,23]. In contrast, reports of high efficiencies of transfection of the rainbow trout gonad (RTG2) cell line are not abundant despite it being the best known trout cell line obtained to date [5,6,24]. Neither transfection of EPC, nor of RTG2 has yet been reported with one of the most recent and promising non-viral transfectant reagents, the widely used mammalian transfectant polyethylenimine (PEI) polymers.

During the last few years, chemically characterized [–HN–CH_2–CH_2–NH–]*n*, high-molecular weight polymers (belonging to the polycationic polymer class) of relatively low cost, polyethylenimines (PEIs) are widely used as vectors for nucleic acid delivery in mammalian cells, being often described as one of the most promising non-viral vectors for gene therapy applications [25–27]. Several different molecular weights (10–40 kDa) [28], linear or branched and/or chemically derived PEI polymers have been tested for DNA/RNA delivery to different mammalian cells [29,30], for instance to *in vitro* validate DNA vaccines [31] and to *in*

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vivo transfer nucleic acids in model animals [32]. In mammal cell lines which grow at 37 °C, intermediate molecular weight PEI polymers (5 kDa) had the highest expression efficiencies (number of cells expressing the transfected gene per number of cells surviving transfection), despite higher molecular weight forms (~8.1 versus 5 and 1.8 kDa) having superior transfection efficiencies (number of transfected cells per initial number of cells exposed to the transfectant) because of their higher cellular toxicities [28].

The first commercial preparations of linear PEI contained residual *N*-acylgroups (*N*-propionyl) as a result of their synthesis procedure. For instance, data obtained by NMR spectrums showed ~10% of *N*-propionyl groups remaining in commercial PEI 25 kDa preparations. More recently, the total removal of the residual *N*-propionyl groups from commercial linear PEI (deacylated PEI or PEI 25 kDa Max®) enhanced their plasmid DNA *in vitro* delivery efficiency by ~20-fold [33]. The transfection improvement seemed to be due to an increase in the number of protonable nitrogens, which presumably results in a tighter plasmid DNA condensation by deacylated PEI and a better endosomal escape of the deacylated PEI–DNA complexes [29], thus diminishing DNA degradation by DNAses [25,28,34].

Because of its high expression efficiency in mammalian cell lines, we have explored the possible use of PEIs of different chemical compositions for transfection on fish cell lines with usual medium (EPC) or very low (RTG2) expression efficiencies. Moreover, due to their low cost, there is a potential use of PEIs to improve the efficiency of fish DNA vaccination against viral pathogens.

Amongst the fish viral pathogens, rhabdoviruses such as viral haemorrhagic septicemia virus (VHSV) constitute one important threat to worldwide aquaculture. Present DNA vaccines against fish rhabdoviruses, including the one for salmonids licensed in Canada in 2005 [35], require their intramuscular delivery by injection. However, oral and/or immersion delivery to obtain *in vivo* transfection throughout epithelial tissues (mass DNA vaccination) will be more practical for aquaculture [11,16,36]. Such mass DNA delivery procedures could benefit from the use of PEI polymers.

Because the *epithelioma papulosum cyprini* (EPC) cell line isolated from carp epithelia [1], is readily transfected [21,22,37,38], we have used EPC cells before as an *in vitro* model [6] to study VHSV-G protein expression [7] and for previous validation of possible fish epithelial DNA vaccine new vectors [16]. We have used the EPC cell line in this work mainly for optimization of PEI transfection. Because the rainbow trout gonad (RTG2) cell line is becoming one of the most useful available salmonid cell lines to study *in vitro* cellular immunology [19,20] and is not readily transfected, we have explored the potential of PEIs to transfect this trout cell line. We report here the transfection of both EPC and RTG2 with similar or better expression efficiencies than with other commercially available transfectant reagents previously used.

2. Materials and methods

2.1. Transfectants

Fugene 6® and Fugene HD® were obtained from Roche (Roche, Barcelona, Spain), Lipofectamine®, lipofectamine + R plus®, lipofectamine 2000® and lipofectin® from Gibco (Gibco BRL, Life Technologies, Gaithersburg, MD, USA), JetPEI® (Polyplus Transfect, Genycell Biotech, Granada) and TransIT LT1® from Mirus (Euromedex, Souffelweyersheim, France). Polylysine was obtained from Sigma (Sigma Che. Co., St. Louis, MO, USA). Commercial linear PEI of 10, 25 and 70 kDa and deacylated PEI of 25 kDa (commercial name PEI 25 kDa Max®) were obtained from Polysciences (Warrington, PA, USA).

2.2. Plasmids

The pMCV1.4 plasmid (Ready Vector, Madrid, Spain) was used as the DNA backbone (1.9 kbp) for the plasmid constructs employed. The MCV1.4 promoter of the pMCV1.4 plasmid is a larger immediate early cytomegalovirus (IE-CMV) promoter of 994 bp which includes a synthetic human globin–immunoglobulin chain intron [38]. The plasmid pMCV1.4-G coding for the protein G of viral haemorrhagic septicemia (VHSV) strain VHSV-0771, isolated in France from rainbow trout (*Oncorhynchus mykiss*) [39] and the pMCV1.4-βgal coding for *Escherichia coli* β-galactosidase and pMCV1.4-EGFP coding for the enhanced green fluorescent protein, were constructed as previously described [7,38].

E. coli DH5alpha (Invitrogen, Barcelona, Spain) were transformed by electroporation with each of the plasmids. Large amounts of plasmids were prepared from *E. coli* pellets by using a modification of the Wizard plus Megaprep DNA purification system (Promega, Madison, USA). The concentration of the DNA was then estimated by fluorescence after SYBR binding (Sigma Che. Co. St. Louis, MO, USA), by diluting the initial solution of SYBR 500-fold in 5 M NaCl, 100 mM $NaPO_4H_2$ pH 7 and measuring fluorescence at 485/535 nm (linearity from 5 to 200 μg DNA/ml). Contamination with proteins was estimated with the help of nanodrop ND1000 spectrophotometry measurements (Nanodrop Technologies Inc, Wilmington, DE, USA). Plasmid solutions adjusted to 0.5–1 mg/ml were kept frozen.

2.3. Transfection of EPC and RTG2 cells

Epithelioma papulosum cyprini (EPC) cells [1] purchased from the European collection of cell cultures (ECACC no. 93120820), and RTG2 (rainbow trout gonad) [2], purchased from the American Type Culture Collection (ATCC CCL 55), were used. EPC and RTG2 were grown in 25 cm^2 flasks at 28 °C/20 °C, respectively, in RPMI Dutch modified/MEM with Earle's salts, respectively, cell culture medium buffered with 20 mM HEPES (Sigma Che. Co, USA) and supplemented with 10% fetal calf serum, 1 mM pyruvate, 2 mM glutamine, 50 μg/ml of gentamicin and 2.5 μg/ml of fungizone and gassed with 5% CO_2 in air.

For the cell transfections, 10 μl of plasmids at different concentrations (2-fold serially diluted in sterile double distilled water starting with 5000 ng/ml) were pipetted in each of the wells of 96-well plates and incubated for 30 min with 25 μl of RPMI medium (Flow) containing 1 μl of commercial transfectants or 250 ng of PEI Max-LoTo per well. EPC or RTG2 cell monolayers were trypsinised (0.05% trypsin, 50 mM EDTA in phosphate buffered saline, PBS), counted with a hemocytometer and adjusted to 500,000 or 120,000 cells per ml, respectively. Then 100 μl of the cell suspension were pipetted into wells containing the plasmid and transfection reagent mixtures. To assay for βgal or EGFP expression, the EPC or RTG2 cell cultures were incubated at 28 °C or 20 °C for 1 or 4 days, respectively. To study VHSV-G protein expression, the EPC or RTG2 cell cultures were incubated at 20 °C for 2 or 7 days, respectively, since these were the optimal conditions for the G protein to be expressed in each of the fish cell lines [21,22,38].

2.4. Fractionation of deacylated PEI (PEI 25 kDa Max®) by Sephadex G50 chromatography

Two ml of 200 mg/ml of deacylated PEI 25 kDa in 50 mM sodium acetate pH 5.5, were applied to a 1.1 × 46 cm column of Sephadex G50 (Pharmacia) equilibrated with the same buffer and fractions of 1 ml were collected. The column was calibrated with blue dextran (Vo), peroxidase (40 kDa), cytochrome *C* (12 kDa) and polyethylene glycol PEGs (4, 1 and 0.7 kDa). Fractions collected based on size

(or elution volume) were separately pooled after the results of their toxicity and expression assays. They were named high toxicity (PEI-HiTo), low toxicity (PEI-LoTo) and no toxicity (PEI-NoTo) fractions.

2.5. Cellular toxicity assays

Two different cytotoxic tests were used to obtain a more convenient and a stronger evidence of cell toxicity, the visual cell detachment and the MTT assays.

The visual cell detachment assay had the advantage that the same assayed wells could then be assayed for transfection assays. Some of the PEI-transfected cell monolayers had only a few cells remaining in the monolayer most being rounded detached cells while others had all their cells in a monolayer as in the controls having no PEI added. Thus, the cellular toxicity of the gel chromatography fractions could be determined by counting the number of cells remaining in the monolayer after 1 day of incubation with pMCV1.4 βgal and 0.15, 0.35 or 0.7 μl of each PEI fraction per well. The mean of the 3 values was used to calculate the number of detached cells per well by the formula, number of initial cells plated − number of cells remaining in the monolayers.

The cytotoxic effects of the PEI-LoTo (from 0 to 1000 ng/well) were also determined by quantifying the EPC and RTG2 cell viability using an MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium] (Sigma, St. Louis, MO, USA) assay. Briefly, assays were performed by adding PEI (0–1000 ng/well) to EPC plated at 40×10^3 cells/well or to RTG2 plated at 12×10^3 cells/well. To determine cellular toxicity of PEI at lower times of exposure, PEI-LoTo containing medium was carefully removed 4 h later from half of the experiments and replaced with fresh medium. Twenty four (EPC) or 144 (RTG2) h later, PEI-LoTo containing medium was carefully removed, 100 μl of MTT (5 mg/ml PBS) diluted 10-fold in fresh cell culture medium added to each well and plates further incubated for 3 h at 28 °C (EPC cells) or 20 °C (RTG2 cells). Finally, the culture medium was again removed and cell viability was measured by the bioreduction of MTT to a colored formazan product that was dissolved in 100 μl/well of DMSO and measured at 570–620 nm with a microplate reader. Absorbance at 620 nm being used to correct for individual variations between wells. Cellular toxicity was calculated by the formula, 1 − absorbance at 570–620 nm of transfected cell monolayers/absorbance at 570–620 nm of control cell monolayers. Percent of viability was calculated by the formula, absorbance at 570–620 nm of transfected cell monolayers/absorbance at 570–620 nm of control cell monolayers × 100. Six replicates per point were used.

2.6. β-Galactosidase activity

The Gal-screen gene assay system (Tropix, Bedford, MA, USA) was used. Briefly, after removing the cell culture medium, 50 μl/well of 0.025% Triton X-100 were added to lyse the cell monolayers and the plates agitated for 5 min. Then, 1 μl of substrate and 25 μl of lysis enhancer buffer mixture were added per well, incubated for 60 min at room temperature and counted for 1 s (counts per second, cps) in a Tecan Genios apparatus (Tecan, Salzburg, Austria).

2.7. EGFP fluorescence

EPC cells (40×10^3 cells/well) or RTG2 (12×10^3 cells/well) were transfected with 0–500 ng/well of pMCV1.4-EGFP and 250 ng of PEI-LoTo. To decrease cellular toxicity, the cell culture medium was removed in EPC at 4 h or in RTG2 at 24 h after the addition of the pMCV1.4-EGFP and PEI-LoTo mixtures. Alternatively, the cell culture medium was not removed after the addition of the pMCV1.4-EGFP and PEI-LoTo mixtures. After 24 h for EPC or 144 h for RTG2, the cell monolayers were lysed with 100 μl of 0.05% Triton X-100 and fluorescence measured at 485 nm/530 nm excitation/emission in a fluorescence microplate reader (Fluorostar Galaxy BMG Labtechnologies, GmbH, Germany). Data were expressed in arbitrary units as the means ± standard deviations of triplicate experiments.

2.8. Anti-G monoclonal antibodies (MAbs) C10, I16 and 3F1A12

The anti-VHSV-G monoclonal antibodies (MAbs) C10 [40,41], I16 (INRA, unpublished) and 3F1A12 (Aarhus, unpublished) were obtained from INRA (Dr. M. Bremont) and Denmark Centre of Aarhus (Dr. N. Lorenzen), respectively. At least 500 ml of hybridoma culture supernatants were obtained, characterized and concentrated-purified by affinity chromatography on protein A. The I16 was mapped in between amino acids 139–153 by using a G pepscan as previously described [42,43]. An equimolecular mixture of C10, I16 and 3F1A12 (C10 + I16 + 3F1A12 mix) at a final concentration of 1 mg/ml of protein was used to detect the protein G of VHSV.

2.9. VHSV-G protein detection by immunodetection and immunofluorescence

We used the VHSV-G assay by transfection with the pMCV1.4 plasmid because it has been characterized in many of our previous publications (FACS, Q-RT-PCR, G-dependent fusion, etc.) [7,44,45]. In this work, the ability of the plasmid pMCV1.4-G to mediate expression of VHSV-G protein in the transfected cells using PEI was confirmed by G-specific monoclonal antibody immunostaining for the viral protein as previously described either by peroxidase or by fluorescence [46].

To recognize the protein G by peroxidase, EPC or RTG2 cells were transfected with pMCV1.4-G as described above and incubated at 20 °C for 2 days or 7 days, respectively (optimal temperature/time for G expression). Then, the cell culture medium was eliminated and the cell monolayers incubated for 60 min with 50 μl of the C10 + I16 + 3F1A12 mix 250-fold diluted in RPMI-10% fetal calf serum. After carefully washing with 50 μl of RPMI-10% fetal calf serum, the cell monolayers were incubated for 30 min with 50 μl of peroxidase-labeled rabbit anti-mouse IgG 500-fold diluted in RPMI-10% fetal calf serum. After washing twice with 100 μl of RPMI-10% fetal calf serum, the cell monolayers were incubated with 50 μl of 0.8 mg/ml of diaminobenzidine (DAB) in PBS containing 1.5 μl per ml of H_2O_2 until brown cells (DAB-positive cells) appeared under the inverted microscope. The percentage of DAB-positive cells were calculated by the formula, number of DAB-positive cells/number of cells × 100.

To recognize the protein G by immunofluorescence, pMCV 1.4-G transfected EPC or RTG monolayers were incubated at 20 °C for 2 days or 7 days, respectively, washed with 0.1% bovine serum albumin (BSA) and 1% rabbit serum in PBS, fixed with cold methanol (15 min at room temperature) and air dried as described before [46]. The fixed monolayers were then incubated with the MAb anti-VHSV-G protein I16 diluted 250-fold in PBS for 4 h at room temperature. After washing the cells with 150 μl of PBS per well, 100 μl of fluorescein-labeled rabbit anti-mouse IgG (Sigma) 300-fold diluted in PBS were added per well and the incubation continued for 45 min. Cells were viewed and photographed with an inverted fluorescence microscope (Nikon) provided with a digital camera (Nikon DS-1QM). The percentage of fluorescent-positive cells was calculated by the formula, number of fluorescent-positive cells/total number of cells × 100.

3. Results

To compare expression efficiencies with different PEI polymers, preliminary results selected Fugene 6® (Roche), Fugene HD® and TransIT LT1® (Mirus) as the commercial transfectants having the highest and more reproducible expression efficiencies in fish cells. Polylysine, Lipofectamine®, lipofectamine + R plus®, lipofectamine 2000® and lipofectin® (Gibco BRL) were discarded (data not shown).

Fig. 1 shows a comparison of the transfection levels obtained by using the pMCV1.4-βgal plasmid in EPC cells with the selected commercially available transfection reagents including commercial PEI (JetPEI®) and various PEI polymers of different molecular weights. Best levels of transfection were consistently obtained by the use of Fugene 6®, Fugene HD® or Transit LT1® in different experiments throughout the years while 3–4- or 10–15-fold lower transfection levels were obtained with JetPEI® and PEI 25 kDa Max® (deacylated PEI 25 kDa) or with PEI of 10, 25 (Fig. 1) and 70 kDa (not shown), respectively. Although variations do exist depending on the experiment, the differences between unfractionated PEIs and the rest of the transfectant reagents were consistently observed.

Because previous observations indicated that the cellular toxicity of PEIs was related to their molecular weights and chemical synthesis produces a wide rather than a narrow distribution of molecular weights, we fractionated deacylated PEI 25 kDa by Sephadex G50 chromatography and assayed each of the fractions obtained for both cellular toxicity and expression efficiencies by complexing each of the fractions with pMCV1.4-βgal and transfecting those to EPC cells. Fig. 2A shows that the nominal 25 kDa molecular weight of deacylated PEI 25 kDa was homogenously distributed in a 40–0.7 kDa range of molecular weights. Cellular toxicity as estimated visually by EPC monolayer cell lysis one day after transfection and confirmed by MTT assays, was increasing from fractions 10–17 (40–20 kDa) and then decreasing from fractions 18–30 (<20–0.7 kDa) (Fig. 2B). After several preliminary assays, the amount of PEI to be included into the assays to obtain any measurable transfection was calibrated to produce a significant βgal expression with a minimal toxicity in most fractions (initial PEI fractions have to be diluted 6-fold and 1 μl of each diluted fraction were sufficient to transfect 250 ng of pMCV1.4-βgal in the conditions defined for the assay). Fig. 2A shows that the profile of βgal expression obtained had a maximum βgal expression at about 15 kDa. Fractions 12–17 (high toxicity, HiTo), 18–21 (low toxicity, LoTo) and 22–27 (no toxicity, NoTo) were then pooled for further analysis and the fractions named PEI-HiTo, PEI-LoTo and PEI-NoTo, respectively.

Similar profiles of cellular toxicity and expression efficiencies were obtained when the PEI fractions of the Sephadex G50 chromatography were assayed in the RTG2 cell line although with a ~5-fold lower expression efficiency (data not shown).

Fig. 3A shows that fractionated-pooled PEI-LoTo and TransIT LT1 had comparable expression efficiencies while fractionated-pooled

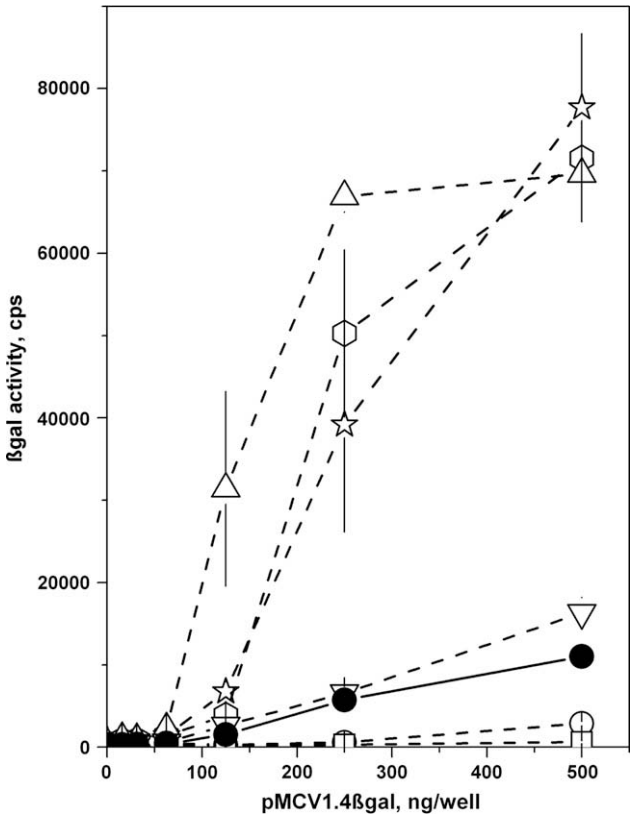

Fig. 1. Comparison of expression efficiencies of EPC cells transfected with different transfection reagents and different unfractionated PEIs. EPC cells were transfected with the pMCV1.4-βgal plasmid with different commercially available transfection reagents: ☆, Fugene 6®. ⬠, Fugene HD®. △, Transit LT1®. ▽, JetPEI®. ○, PEI 10 kDa. □, PEI 25 kDa. ●, deacylated PEI 25 kDa (PEI 25 kDa Max®). Expression efficiencies obtained with linear PEI 70KDa were similar to those obtained with PEI 10-25 kDa (not shown). Mean and standard deviations from two experiments are represented.

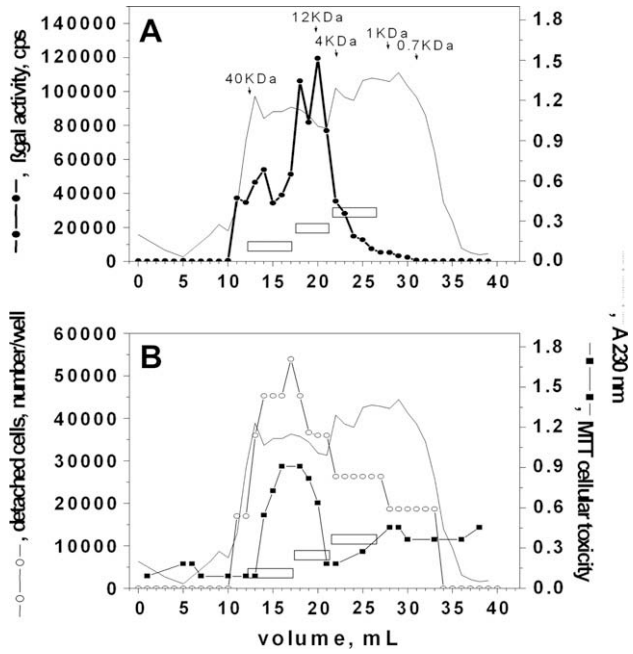

Fig. 2. Fractionation of deacylated PEI 25 kDa (PEI 25 kDa Max®) by Sephadex G-50 chromatography and assay of the fractions transfected into EPC cells by β-galactosidase expression (A) and cellular toxicity (B). Two ml of 200 mg/ml of deacylated PEI 25 kDa Max® in 50 mM sodium acetate pH 5.5, were applied to an 1.1 × 46 cm column of Sephadex G50 (Pharmacia) equilibrated with the same buffer and fractions of 1 ml were collected. Each of the fractions was 6-fold diluted with distilled water and 1 μl assayed by transfection of EPC cells by using the pMCV1.4 βgal plasmid. Results show the average of the results of two experiments, standard deviations omitted for clarity. The column was calibrated (vertical arrows) with blue dextran (Vo), peroxidase (40 kDa), cytochrome *c* (12 kDa) and polyethylene glycol PEGs (4, 1 and 0.7 kDa). Fractions 12–17 (high toxicity, HiTo), 18–21 (low toxicity, LoTo) and 22–27 (no toxicity, NoTo) ml were pooled (horizontal open rectangles). ●, β-galactosidase activity. ……A 230 nm, absorbance of the PEI fractions (1 mg/ml of PEI had an A_{230nm} of 0.2). ○, number of detached cells per well as calculated by the formula, number of initial cells plated – number of cell remaining in monolayer after 1 day. ■, MTT cellular toxicity was calculated by the formula, 1 – absorbance at 570–620 nm of EPC transfected monolayers/absorbance at 570–620 nm of control EPC monolayers.

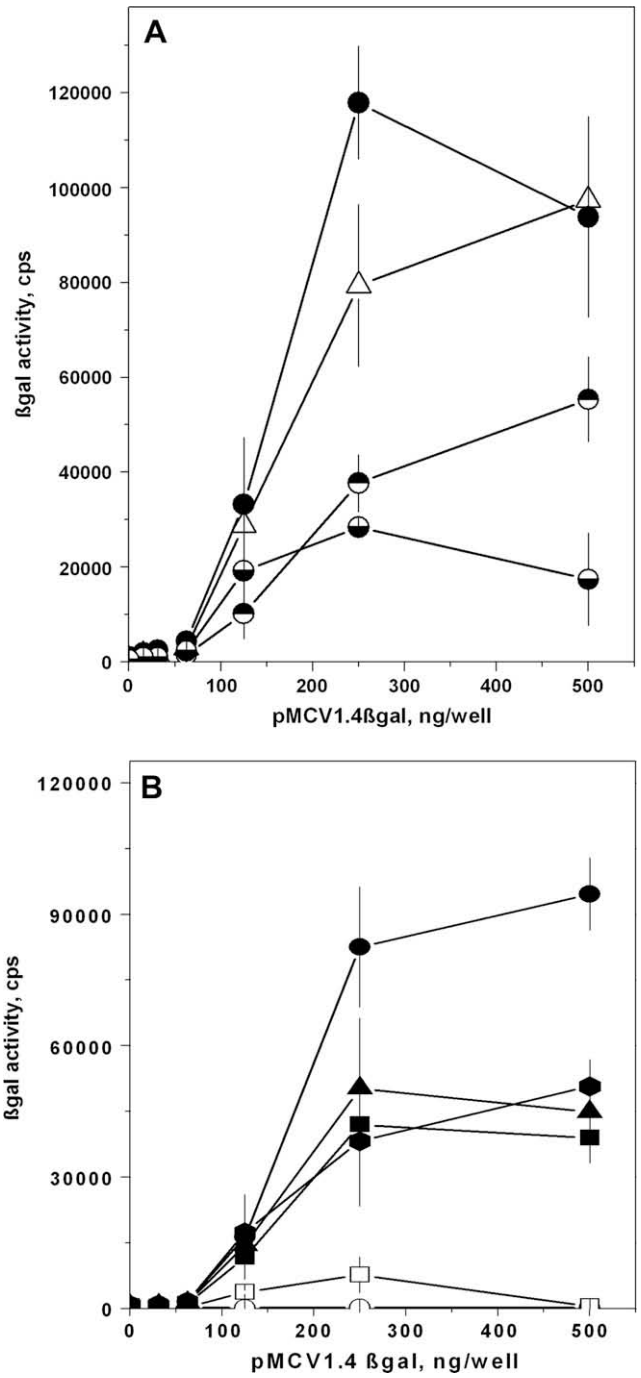

Fig. 3. β-galactosidase activity in EPC cells transfected with different amounts of pooled fractions of chromatographed PEI 25 kDa Max® (A) and with different concentrations of PEI-LoTo (B). EPC cells were transfected with pMCV1.4 βgal and different transfectant reagents as indicated in methods. (A) ●, PEI-LoTo. ◓, PEI-HiTo. ◒, PEI-NoTo. △, Transit LT1. (B) EPC cells transfected with different concentrations of PEI-LoTo: ○, 50 ng /well. □, 100 ng/well. ■, 200 ng/well. ▲, 250 ng/well. ●, 300 ng/well and ⬟, 400 ng/well. Average and standard deviation from at least 3 experiments are represented.

PEI-HiTo and PEI-NoTo were 3–4-fold lower thus confirming the results obtained by the individual (Fig. 2) rather than the pooled PEI fractions. Fig. 3A and B also shows the results from two different ways to study the influence of transfectant/DNA ratios on expression efficiency. Thus while Fig. 3A shows the results obtained by using a constant amount of every transfectant with 6 different DNA concentrations of pMCV1.4 βgal from 0 to 500 ng/well (resulting in 6 different transfectant/DNA ratios), Fig. 3B shows the results obtained by using constant amounts of DNA and varying concentrations of PEI-LoTo. Similar and optimal transfections were obtained by using any of those two methods when 250–300 ng of PEI-LoTo were complexed with 300–500 ng of DNA per well in a 96 wells plate.

To try to increase transfection/expression by reducing cellular toxicity, the exposure time of the cells to the PEI-LoTo reagent was reduced from 24 to 4 h at several transfectant/DNA ratios. Also, other reporter gene, coded in the pMCV1.4-EGFP plasmid was used for the transfection assays to corroborate the results obtained before with the pMCV1.4-βgal. Exposures of 4 h to the PEI-LoTo increased the amount of PEI-LoTo cells that could resist <50% lysis from ~250 to 500 ng per well in EPC and from ~400 to 1000 ng per well in RTG2 (Fig. 4A). However, the profiles of expression efficiency/amount of pMCV1.4-EGFP were not significantly improved in any of the cell lines (Fig. 4B). The hardest-to-transfect cell line RTG2 in these experiments showed a slightly higher expression of EGFP than EPC (Fig. 4B). Fig. 4C and D shows the appearance of EPC and RTG2 monolayers after transfection with pMCV1.4-EGFP both at the visible and fluorescence microscope. Similar results were obtained by using the βgal reporter system (not shown).

EPC and RTG2 cell monolayers were then transfected with the well characterized pMCV1.4-G plasmid coding for the glycoprotein G of VHSV [7,44,45] at several transfectant/DNA ratios to test the possible use of PEI-LoTo in assays of protein G expression to optimize vector constructs for DNA vaccination [16] or to assay for specific cell-mediated antiviral cytotoxicity [19,20]. Because the temperature in these assays cannot be higher than 20 °C due to denaturation of the glycoprotein G [7], the performance of PEI-LoTo at that temperature was not necessarily deduced from their performances at 28 °C. Fig. 5A shows, that protein G expression could be demonstrated by immunofluorescence assays in both EPC (about 30% expression efficiency) or RTG2 (about a 6% expression efficiency) cell lines with a similar maximal extent as that obtained with Fugene 6® and/or FuGeneHD® reagents in each case. Fig. 5B and C shows the appearance of EPC and RTG2 monolayers after transfection with pMCV1.4-G both at the visible and fluorescence microscope. Similar results relative to Fugene 6® and/or FuGeneHD® reagents, although with apparently lower expression efficiencies, were obtained by using immunoperoxidase assays instead of immunofluorescence (not shown).

4. Discussion

Polycations such as polylysine have been used for the introduction of foreign DNA into mammalian cells (grown at 37 °C) long before the use of liposomal formulations [47]. However, progress was slow until the introduction of polyethylenimine (PEI) in 1995. Polycations were scarcely used before to transfect fish cells which are grown at lower temperatures (15–28 °C) [6,47].

Although in the first fish transfection assays reported here, the relative expression efficiencies were low for PEI of 10, 25 or 70 kDa of molecular weights (Fig. 1) and for polylysine (results not shown), the use of PEI 25 kDa Max® (deacylated PEI 25 kDa) after removal of their molecular weight components of higher cellular toxicity (PEI-LoTo) resulted in fish cell transfection levels as high as those obtained with previously used commercially available transfection reagents (Fig. 3). In our hands, not only EPC (grown at 20 or 28 °C) but also difficult-to-transfect fish cell lines, such as RTG2 (grown at 20 °C), could be transfected with PEI-LoTo.

Possible relationships between PEI size, toxicity and transfection/expression efficiencies similar to those made previously in CHO cells at 37 °C [28] can be made from the results obtained in this

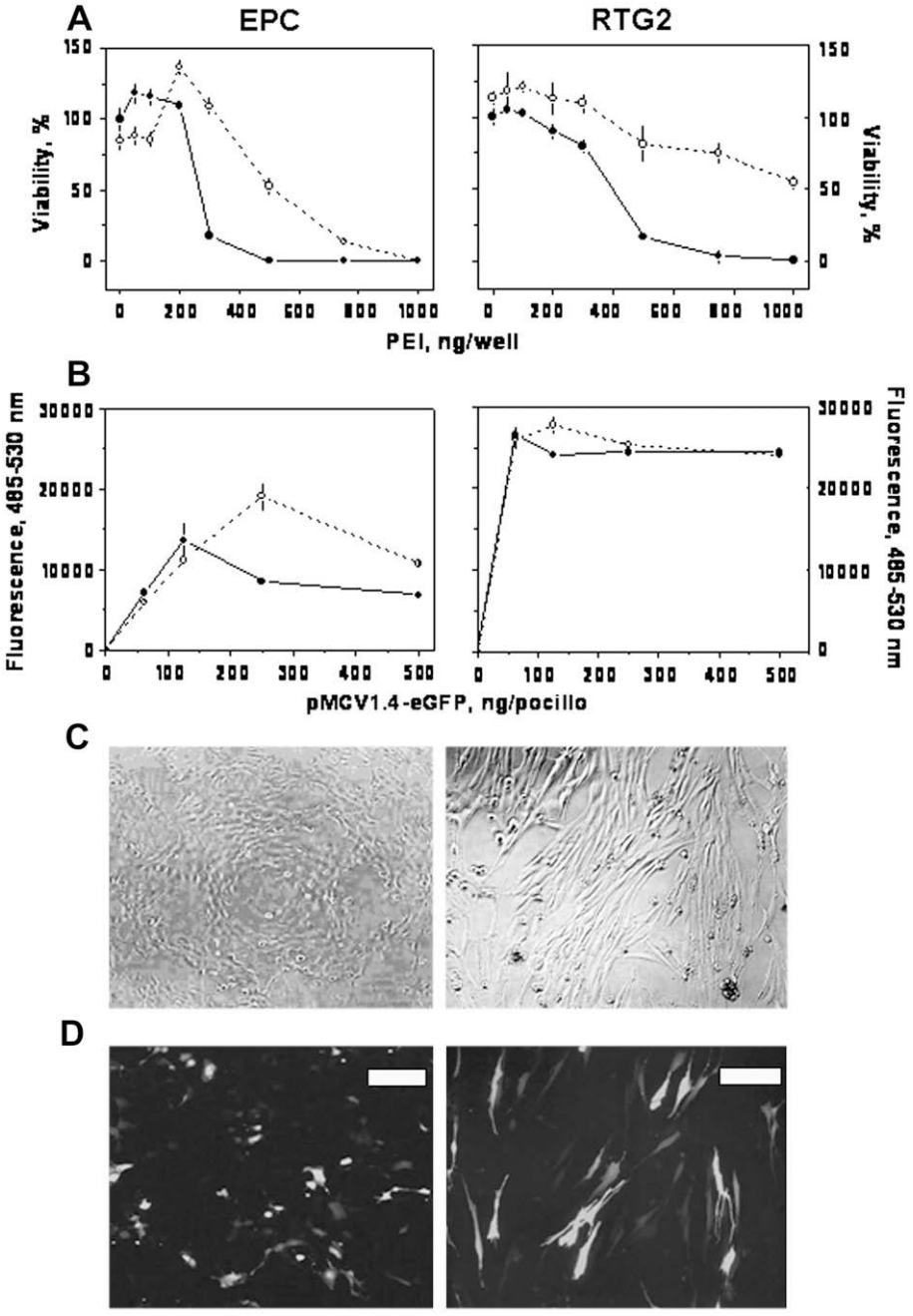

Fig. 4. Cellular toxicity assays of PEI-LoTo (A), expression of EGFP (B) and micrographs at visible (C) and fluorescent (D) light fields in EPC and RTG2 cells. (A) PEI-LoTo (0–1000 ng/well) was added to cell monolayers and assayed for cellular toxicity by the MTT viability assay after 24 h in carp EPC or after 144 h in trout RTG2. To decrease cellular toxicity, the cell culture media was removed in EPC at 4 h or in RTG2 at 24 h after the addition of the PEI-LoTo (○). Alternatively the cell culture media was not removed after the addition of PEI-LoTo (●). Percent of cellular toxicity was determined by the MTT viability assay and calculated by the formula, absorbance at 620 nm of EPC or RTG2 PEI-LoTo transfected monolayers/absorbance at 620 nm of control EPC or RTG2 monolayers × 100. Data are expressed as means ± standard deviations of six determinations per point. (B) EPC cells (40×10^3 cells/well) and RTG2 (12×10^3 cells/well) were transfected with 0–500 ng/well of pMCV1.4-EGFP and 250 ng of PEI-LoTo. After 24 h (EPC) or 144 h (RTG2) of transfection, cell monolayers were lysed and EGFP fluorescence measured at 485 nm/530 nm. Data are expressed as arbitrary units as means ± standard deviations of triplicate determinations. (C and D) Visible and fluorescent fields, respectively, of microphotographs of EPC and RTG2 cell monolayers transfected with pMCV1.4-EGFP with PEI-LoTo complexes as above. Microphotographs were taken under the phase contrast and fluorescence microscopy 24 h (EPC) or 144 h (RTG2) after transfection. The horizontal bar is 50 μm.

work in EPC and RTG2 cells at 28 and 20 °C, respectively. Thus, although Fig. 1 shows very small non-significant expression efficiencies for both PEI 10 kDa and PEI 25 kDa, fractionation of PEI 25 kDa Max® (Fig. 2), showed that optimal expression efficiencies were at ~15 kDa (higher or lower molecular weights having either high toxicities or low expression efficiencies, respectively). Pooled fractions (Fig. 3A) of the higher molecular weights (~40 kDa) have lower expression efficiencies most probably due to their higher cellular toxicity despite having high transfection efficiencies (PEI-HiTo) while those having lower molecular weights (~4 kDa) have also lower expression efficiencies most probably due to their lower transfection efficiencies despite their lower toxicity (PEI-NoTo), thus confirming the expression efficiency results obtained by fractionation of PEI 25 kDa Max® (Fig. 2). An optimal equilibrium between cellular toxicity and transfection efficiency resulted in optimal expression efficiencies shown by fractions around 15 kDa

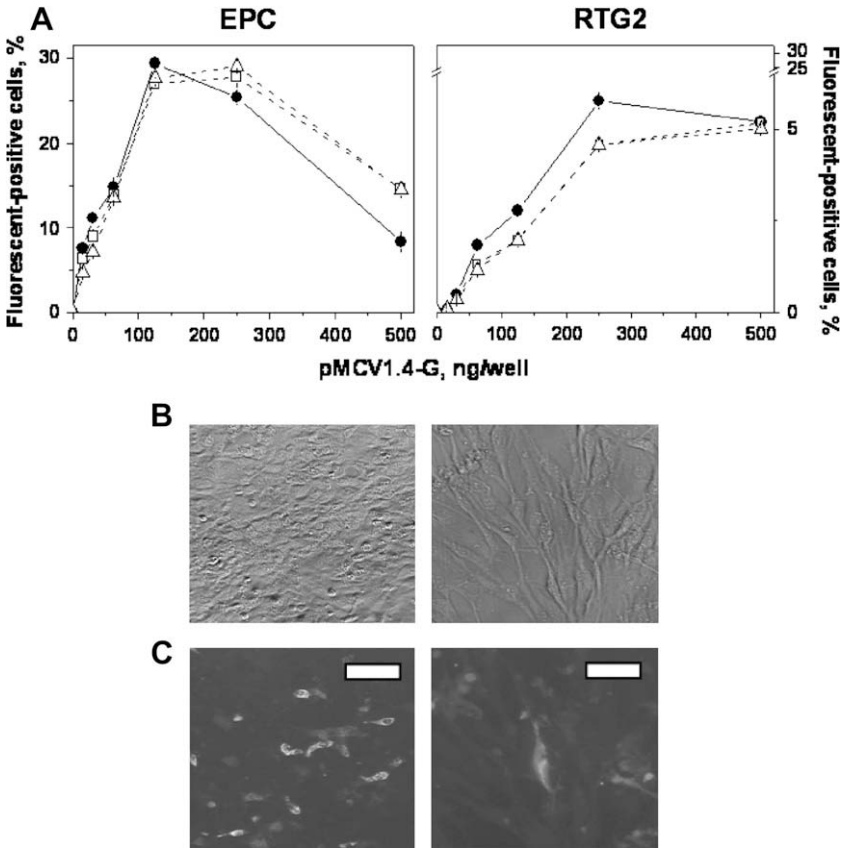

Fig. 5. Expression of protein VHSV-G protein in EPC and RTG2 transfected cells using PEILoTo(●), FuGene6 (□) or FuGeneHD (△) reagents. (A) EPC and RTG2 cell lines were transfected with different concentrations of pMCV1.4-G plasmid complexed with PEI-LoTo, FuGene6 or FuGeneHD. After 48 h (EPC) or 144 h (RTG2) of incubation, the cell monolayers were washed, fixed and then stained. (B and C) Microphotographs of EPC and RTG2 cell line monolayers transfected with pMCV1.4-G PEI-LoTo complexes. The horizontal bar is 50 μm. Average and standard deviations from three experiments are represented.

(PEI-LoTo). Similarly, in CHO cells, although 8.1 kDa PEI polymers had superior transfection efficiencies than 5 and 1.8 kDa, they also cause higher cellular toxicities, thus resulting in intermediate molecular weights (5 kDa) having the highest expression efficiencies [28].

The higher expression efficiency of PEI compared to other polycations (poly Lys, protamine, etc.), seems to be due to its proton sponge effect [34]. The accepted explanation being that partially protonated polycations absorb protons increasingly during the later endocytic vesicle steps [47], thus causing their rupture and diminishing the degradation of their transported DNA during their endogenous cell pathways. However, as most transfection reagents, excess of concentration of PEI-LoTo could also cause excessive cell death [28] and thus reduce the expression efficiency as demonstrated for both EPC and RTG2 fish cell lines (Fig. 4). Therefore it might be anticipated that any new procedure which will decrease PEI-LoTo toxicity without decreasing transfection efficiency could be used to increase their expression efficiencies on fish cells but that has to be proven.

The deacylated PEI-LoTo could transfect DNA in a wide temperature range. Thus, in the cases of RTG2 and of protein G assays in EPC, the PEI-LoTo was capable of transfecting different gene coding plasmids at 20 °C and at 28 °C. Preliminary results showed PEI-LoTo was also capable of transfecting mammalian cells such as Vero at 37 °C (not shown).

Possible applications of this easily available reagent, new to the fish field, include their use to transfect EPC monolayers with the pMCV1.4-G plasmid coding for the glycoprotein G of VHSV, a method which is required for many different *in vitro* assays [7,8,16,38]. PEI-LoTo could also be used for many other possible *in vitro* assays as mentioned in the Introduction including specific cell-mediated antiviral *in vitro* assays [19,20] and *in vivo* applications such as for DNA vaccines [31]. Furthermore, because its chemical formula is well known and its supramolecular structure can be studied [28–30], it should be available for further chemical manipulations to increase its applicability to different needs in the fish immunology field.

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