International Journal of Pharmaceutics 403 (2011) 276–284

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

# Photochemical internalization for pDNA transfection: Evaluation of poly(D,L-lactide-co-glycolide) and poly(ethylenimine) nanoparticles

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ARTICLE INFO

*Article history:*
Received 16 July 2010
Received in revised form 20 October 2010
Accepted 21 October 2010
Available online 29 October 2010

*Keywords:*
Gene delivery
Nanoparticles
Photochemical internalization
Poly(D,L-lactide-co-glycolide)
Polyethylenimine

ABSTRACT

The main objective of this study was to prepare two types of nanoparticles with poly(D,L-lactide-co-glycolide) (PLGA) and polyethylenimine (PEI) polymers. Plasmid DNA (pDNA) was adsorbed either on PLGA/PEI nanoparticles, or as PEI/DNA complex onto the surface of PLGA nanoparticles. Both types of nanoparticles were prepared by the double emulsion method. The nanoparticles were characterized by their size, zeta potential and pDNA or PEI/DNA complex adsorption. The PEI/DNA complex adsorption was confirmed with ethidium bromide assay. pDNA adsorption onto PLGA/PEI nanoparticles (PLGA/PEI–DNA) was studied by electrophoresis on agarose gel. Cytotoxicity and transfection efficiency of both types of nanoparticle and PEI/DNA complexes formulations were studied in head and neck squamous carcinoma cell line (FaDu). To improve endosomal release, photochemical internalization (PCI) was used. The zeta potential increased when the PEI/DNA complex adsorbed onto PLGA nanoparticles (PLGA–PEI/DNA). Optimal pDNA adsorption efficiency was achieved for nitrogen/phosphorous ratio $\geq$ 20/1. *In vitro* transfection and cells viability on FaDu cells with or without PCI were found to be variable depending on the type and concentration of nanoparticles. The results showed that transfection efficiency for PLGA/PEI–DNA or PLGA–PEI/DNA nanoparticles ranged between 2 and 80%, respectively. PCI was found to slightly improve the transfection efficiency for all formulations.



## 1. Introduction

Gene therapy is a promising technology for the treatment of genetic diseases. Most studies used viral vectors due to their high transfection efficiency (Liu et al., 2010; Wang et al., 2010; Wolfe et al., 2010). However toxicity, immunogenicity, including mutagenesis issues may limit the use of viral vectors. Recently, the use of non-viral vectors such as cationic polymers (O'Rorke et al., 2010), liposomes (Oliveira et al., 2009; Xiao et al., 2010) and nanoparticles (Fay et al., 2010; Yang et al., 2010) to deliver plasmid DNA (pDNA) has been developed as an alternative strategy with less immunogenicity. In addition non-viral vectors are much easier to produce in large quantities (Niven et al., 1997). Among non-viral vectors, nanoparticles have been developed for their multiple advantages in drug delivery. When coating or ligand materials are attached at the surface of biodegradable nanoparticles, higher stability, and easier uptake into cells by endocytosis, and targeting ability to specific tissues or organs, has been reported.

One of the most popular biodegradable polymer is poly(D,L-lactide-co-glycolide) (PLGA). PLGA has been approved by the Food Drug Administration for transfection trials in humans (Budker et al., 1996; Sahoo et al., 2002). Previous studies have demonstrated the potential of PLGA nanoparticles to deliver genes into cells (Fay et al., 2010; Niu et al., 2009; Son and Kim, 2010). Following cells endocytosis, PLGA nanoparticles escape rapidly from the degradative endo-lysosomal compartment to the cytoplasmic compartment (Panyam et al., 2002). The degradation time of PLGA can be altered from several days to years by varying the polymer molecular weight, the lactic acid to glycolic acid ratio in copolymer, or the nanoparticles structure. pDNA could be encapsulated (Cohen et al., 2000) or adsorbed onto nanoparticles (Kumar et al., 2004; Rhaese et al., 2003).

In order to achieve an efficient transfection, the addition of a cationic polymer, e.g. polyethylenimine (PEI) which is commonly used for non-viral pDNA delivery systems, to the PLGA nanoparticles was already investigated (Bivas-Benita et al., 2004; Katas et al., 2009; Park et al., 2008). Adsorption is based on the ionic interactions between the cationic polymers and the anionic pDNA (Singh et al., 2000). PEIs are polymers available in two forms i.e. linear and

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doi:10.1016/j.ijpharm.2010.10.040

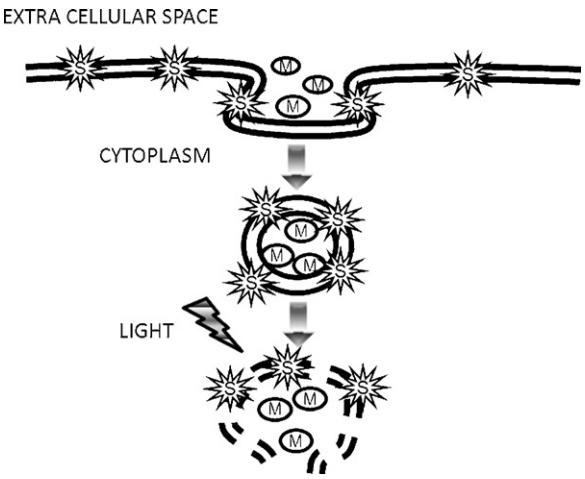


**Fig. 1.** Photochemical internalization mechanism: a photosensitiser (S) and the macromolecule of interest (M) are endocytosed and co-localised to endocytic compartments. Light exposure of the cells introduces rupture of the membrane of these vesicles and subsequent cytosolic release of macromolecules.

branched. Compared to linear forms, branched PEI has shown a better cells rate transfection (Godbey et al., 1999). PEIs exhibit a high positive charge density when protonated in aqueous solutions. The high positive charge density of PEI causes an electrostatic interaction with anionic charged species such as pDNA to form polyplexes. Furthermore, PEI protected pDNA from the degradation of nuclease activity (Kim et al., 2007).

Photochemical internalization (PCI) is a promising technology for site-specific release of macromolecules within cells. The mechanism of PCI is based on the breakdown of the endosomal/lysosomal membranes by photoactivation of photosensitizers that localize in the membranes of the endosomes (Berg et al., 1999) as shown in Fig. 1. This technology has been used to release several macromolecules such as bleomycin (Berg et al., 2005), gelonin (Dietze et al., 2003), DNA as a complex with cationic polymers (Ndoye et al., 2004b) or incorporated in adenovirus (Bonsted et al., 2004) and nucleic acid (Folini et al., 2003), from endocytic vesicles to the cytosol. PCI can significantly increase the transfection efficiency of PEI/DNA complex *in vitro* (Ndoye et al., 2004a), and *in vivo* (Ndoye et al., 2006).

In this study, two types of nanoparticles were evaluated to deliver pDNA into human head and neck squamous carcinoma cell line (FaDu). An optimisation of photochemical internalization conditions was made in a previous study onto this cell line (Ndoye et al., 2004b). PLGA nanoparticles incorporating PEI were prepared using a double emulsion method and the resulting nanoparticles were used to adsorb pDNA for the cell transfection study. In addition, unloaded PLGA nanoparticles were prepared, and PEI/DNA complexes were adsorbed onto their surface. Both types of nanoparticles were characterized with regards to their size, zeta potential, adsorption as well as evaluation of transfection efficiency and cytotoxicity into FaDu cells. Furthermore, the effects of PCI on pDNA transfer in human cancer cells were evaluated using PLGA/PEI or PLGA nanoparticles.

## 2. Materials and methods

### 2.1. Materials

pEGFP-C3 plasmid DNA coding for Green Fluorescent Protein (GFP) was obtained from Clontech (Montigny le Bretonneux, France). The following polymers were used as received: poly(D,L-lactide-co-glycolide) (PLGA 50:50; Resomer RG® 503H, viscosity 0.42 dl/g, uncapped; Boehringer Ingelheim, Ingelheim, Germany) and polyethylenimine (PEI 25 kDa, branched; Sigma–Aldrich, Saint-Quentin Fallavier, France). The poly(vinyl alcohol) (PVA, $M_w$ 30,000, 99% hydrolyzed) was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). The human head and neck squamous carcinoma cell line (FaDu) was kindly provided by Professor A. Hanauske (Munich University, Germany) (ATCC number HTB-43™). Roswell Park Memorial Institute 1640 (RPMI 1640) cell culture medium and Lipofectamine™ 2000 (transfection reagent, a cationic lipid), were purchased from Invitrogen Life Technology (Cergy-Pontoise, France). Disulfonated meso-tetraphenylporphine ($TPPS_{2a}$) (LumiTrans®) was used as photosensitizer and was kindly provided by PCI Biotech (Oslo, Norway). Lumisource® was used as light source.

### 2.2. Preparation of PLGA/PEI and PLGA nanoparticles

The preparation method of PLGA/PEI or PLGA nanoparticles was a modification of the double emulsion method described by Park et al. (2008). Briefly, 4 ml of an organic phase containing PLGA (50 mg/ml in methylene chloride) were magnetically stirred during 15 s with 1 ml of an aqueous solution of PEI at different concentrations (5, 10, 20, 50, 100 or 200 mg/ml) or plain water (1 ml). This emulsion was poured into 16 ml of PVA aqueous solution (5%, w/v), and sonicated (80 W for 30 s) using an ultrasonic homogenizer (Vibracell 75022, Bioblock, Illkirch, France). The solvent was then evaporated under vacuum using a rotary evaporator at 40 °C for 1 min (Heidolph 94200, Bioblock, Illkirch, France). The resulting nanoparticles were obtained by centrifugation (42,000 × *g*, 20 min, 20 °C). The pellet was washed three times with purified water and was re-suspended in 2 ml of purified water. The obtained nanoparticles suspensions were stored at 4 °C.

### 2.3. pDNA preparation

pDNA, pEGFP-C3 encoding the green fluorescent protein (GFP) was propagated into *E. coli* bacterial strain. Transformed cells were grown in Lysogeny Broth (LB) medium (Tryptone, yeast extract and NaCl). Plasmid was purified using Marligen maxiprep™ high purity plasmid purification columns (Clinisciences, Montrouge, France). The pDNA concentration was determined by UV spectrophotometry (Uvikon 922, Kontron, Eching, Germany) at 260 nm and the purity was confirmed by using 0.8% agarose gel electrophoresis (Bio-Rad, Marnes-la-Coquette, France) in the presence of ethidium bromide (EtBr).

### 2.4. PEI/DNA complexes preparation and adsorption of pDNA or PEI/DNA complexes onto nanoparticle surface

PEI/DNA complexes were prepared according to various nitrogen/phosphorous (N/P) ratios. The PEI polymer concentrations were calculated from the desired N/P ratio and the amount of plasmid assuming that 43.1 g/mol$^{-1}$ corresponds to each repeating unit of PEI containing one nitrogen atom, and 330 g/mol$^{-1}$ corresponds to each repeating unit of pDNA containing one phosphorous atom. The resulting charge ratio is expressed as PEI nitrogen/DNA phosphorous (Ho et al., 2008).

Different N/P ratios (from 5 to 20; PEI or PLGA/PEI nanoparticles) were adsorbed with a fixed amount of pDNA. Briefly, 4 μg of DNA in 5% glucose solution were mixed with different amounts of PEI or PLGA/PEI nanoparticles (according to the tested N/P ratio) also diluted in 5% glucose solution. PEI solution or PLGA/PEI nanoparticles suspension (50 μl) was added to the pDNA solution (50 μl), mixed by vortexing for 10 s and incubated for 30 min at room temperature. Attention has to be taken to the nomenclature used in this manuscript: Blank PLGA nanoparticles adsorbed

with the PEI/DNA complexes are called PLGA–PEI/DNA nanoparticles whereas PLGA/PEI nanoparticles adsorbing the naked pDNA are called PLGA/PEI–DNA nanoparticles. For PLGA–PEI/DNA nanoparticles, the PEI/DNA complexes (N/P = 20), prepared in advance as mentioned above, were added to different concentrations of blank PLGA nanoparticles suspensions (from 1.3 to 29.8 mg/ml) and were incubated for 1 h at room temperature.

The adsorption efficiency of pDNA (alone or complexed with PEI) adsorbed onto PLGA nanoparticles was obtained by measuring the extinction fluorescence with ethidium bromide (EtBr). After adsorption, nanoparticles were centrifuged at 42,000 × *g* and 100 μl of supernatant was collected. Tris–HCl (80 μl) and 20 μl of EtBr (100 μg/ml) were added into each well of a black plate. Fluorescence studies were performed using a fluorescence spectrometer Synergy 2 (Biotek Instruments GmbH, Colmar, France) at an excitation wavelength of 524 nm and emission wavelength of 582 nm.

### 2.5. Particle size and zeta potential

The size of the nanoparticles and zeta potential were measured with a Zetasizer 3000E (Malvern Instruments, Worcestershire, UK). All the analyses were conducted in triplicate and the results are expressed as means of the three measurements.

### 2.6. Agarose gel electrophoresis of pDNA

The amount of pDNA bound onto PLGA/PEI nanoparticles or complexed with PEI was determined by the electric mobility of pDNA into a 0.8% agarose gel. Different N/P ratios (1/1; 3/1; 5/1; 20/1; 40/1; 50/1 ratios between PLGA/PEI nanoparticles and pDNA) were tested with a fixed amount of pDNA (4 μg). Electrophoresis was carried out at a constant voltage of 90 V (Bio-Rad®, Marnes-la-coquette, France) for 30 min in TBE buffer (4.45 mM Tris-base, 1 mM sodium EDTA, 4.45 mM boric acid, pH 8.3) containing 0.5 μg/ml EtBr. The gels were visualised under a UV transluminator (Geldoc 2000, Bio-Rad®, Marnes-la-Coquette, France).

### 2.7. In vitro release

Release study of either PEI/DNA complexes or pDNA adsorbed onto nanoparticles was determined. Both types of nanoparticles suspension (200 μl) were incubated in 200 μl of Tris–HCl buffer (pH 7.4), in a shaking incubator at 37 °C. Nanoparticles were centrifuged for 20 min at 42,000 × *g* and supernatants were taken periodically. Then, nanoparticles were resuspended and reincubated in fresh Tris–HCl buffer (pH 7.4) (400 μl) till the next sampling time. Supernatants were analysed using EtBr assay as previously described in Section 2.4.

### 2.8. In vitro photochemical transfection

$10^5$ cells per well, were seeded in 24-well culture plates at 37 °C in a 5% $CO_2$ in humidified atmosphere for 24 h to reach 70% confluence at the time of transfection. Then, for the photochemical transfection study the cells were incubated for 18 h with 0.3 μg/ml of $TPPS_{2a}$ and kept in the dark until the end of the protocol. LumiTrans® was dissolved in dimethylsulfoxide (Sigma Aldrich, Saint-Quentin Fallavier, France) as a 350 μg/ml stock solution.

Subsequently, cells were washed twice with phosphate buffered saline (PBS) and supplemented with 0.5 ml of fresh serum-free RPMI medium. The formulations and Lipofectamine™ 2000 were added to the cells and homogenized. FaDu cells were incubated at 37 °C in a 5% $CO_2$ atmosphere for 2 h. Cells were submitted 30 s to blue light emitted by Lumisource® and fresh medium composed of 20% FBS (500 μl) was added. Lumisource® is a bank of 4 fluorescent tubes designed to provide homogeneous illumination of the treatment area, emitting mainly blue light with a peak at 420 nm. The light emission from the illumination area was measured and showed a high homogeneity with a fluence rate of 10 ± 0.1 mW/cm$^2$ over the light field. After 24 h, the cells were washed with PBS and incubated with 200 μl of trypsin/EDTA (0.05%) for 5 min at 37 °C. The cells were obtained in 2 ml of complete fresh medium and centrifuged at 430 g for 10 min at 20 °C. In a final step, pellets were dispersed in PBS and cells were analysed for GFP expression using flow cytometery (FACS Calibur, BD FACS Calibur™, Becton Dickinson, Le Pont-De-Claix, France) (488 nm excitation, 510–520 nm band-pass filter emission). Fluorescence parameters from $10^4$ cells were acquired and analyzed using Cell Quest® software program (Becton Dickinson, Le Pont-De-Claix, France).

### 2.9. Cytotoxicity assay

The cytotoxicity of both types of nanoparticles (PLGA–PEI/DNA or PLGA/PEI–DNA), the PEI/DNA complexes and the Lipofectamine™ 2000 was evaluated using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT colorimetric test. The test is based on the ability of living cells to reduce the water-soluble yellow dye, MTT, to purple water-insoluble formazan product by mitochondrial enzyme succinate dehydrogenase. FaDu cells, seeded in 24-well culture plates ($10^5$ cells/well), were washed twice with PBS at 37 °C. For the photochemical cytotoxicity study, $TPPS_{2a}$ photosensitizer was added (0.3 μg/ml) 18 h before adding the nanoparticles suspensions. Increasing concentrations of nanoparticles or PEI/DNA complexes were added to the cells in FBS free medium and homogenized. FaDu cells were further incubated at 37 °C in a 5% $CO_2$ atmosphere for 2 h. PCI was performed and cells were reincubated for 2 h. Then, 500 μl of 20% FBS complete medium was added in each well for 24 h. Thereafter, cell viability was determined by the MTT assay according to the procedure described by (Ndoye et al., 2004a). Briefly, 250 μl of MTT solution (2.5 mg/ml in PBS) was added in each well and plates were incubated at 37 °C for 4 h. The formed formazan crystals were dissolved using a 25% aqueous solution of sodium dodecyl sulfate (250 μl/per well). The absorbance was measured at 570 nm using a multiwell-scanning spectrophotometer EL 800 universal microplate reader (Biotek Instruments GmbH, Colmar, France). Cell viability was determined as a percentage of absorbance relative to untreated cell.

### 2.10. Statistical analyses

Statistical analyses were performed using a Mann–Whitney *U*-test. Data are presented as means ± standard deviation (SD). $p < 0.05$ was considered as statistically different.

## 3. Results

### 3.1. Particle size and zeta potential of nanoparticles

Blank PLGA nanoparticles diameter was around 380 nm (±10 nm). When prepared with PEI the PLGA/PEI nanoparticles size decreased slightly 322 nm (±14 nm) as the amount of PEI increased. Different concentrations of PEI were tested, and the nanoparticles size decreased only slightly: 377 (±11 nm), 369 (±8 nm), 360 (±9 nm), 335 (±9 nm), 350 (±13 nm), 322 (±14 nm), for 5, 10, 20, 50, 100, 200 mg/ml PEI concentrations, respectively. Zeta potential of PLGA/PEI nanoparticles was determined according to the quantity of PEI added at the beginning of preparation. As shown in Fig. 2a, zeta potentials of PLGA/PEI nanoparticles were positive, ranging from +40 (±2 mV) to +42 (±3 mV) for 5 mg/ml and 200 mg/ml of PEI, respectively whereas negative values were observed for blank PLGA nanoparticles (−26 ± 2 mV) and for PLGA/PEI nanoparticles

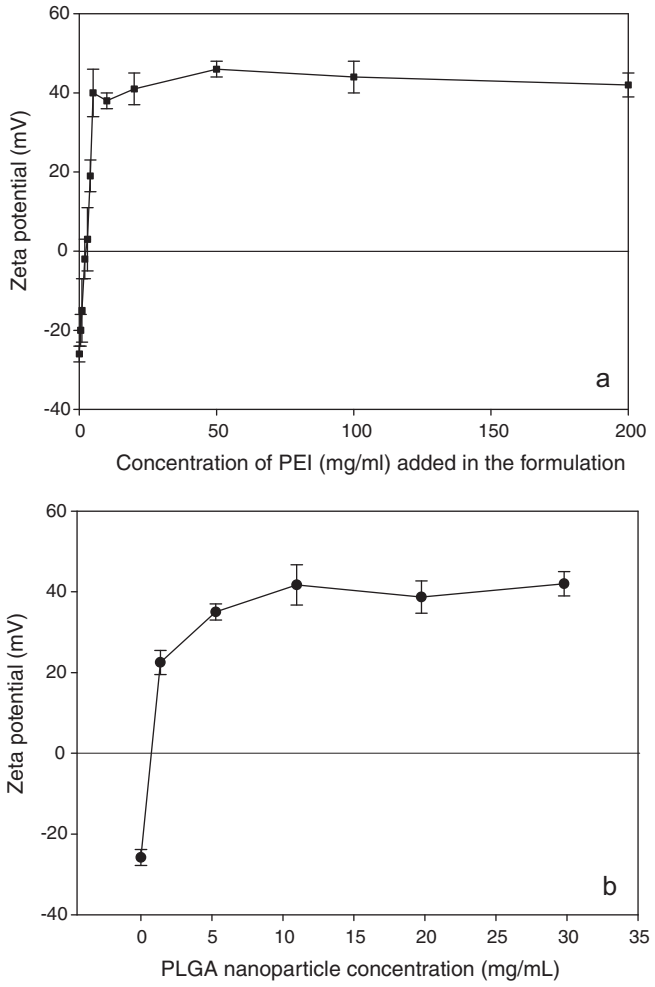


**Fig. 2.** (a) Influence of increasing PEI concentrations on zeta potential of PLGA/PEI nanoparticles ($n = 3 \pm SD$). (b) PLGA nanoparticles binding efficiency and adsorption capacity to adsorb PEI/DNA N/P = 20 complex. PEI/DNA complex (N/P = 20) were prepared, then incubated with PLGA nanoparticles and zeta potential was measured ($n = 3 \pm SD$).

with PEI concentrations from 0.5 to 2 mg/ml. For the following studies, we decided to formulate nanoparticles with a PEI concentration of 5 mg/ml. The particles size of PLGA/PEI–DNA nanoparticles (5 mg/ml of PEI) prepared with 5, 10, 20 N/P ratios were 380 nm ± 17 nm, 368 ± 20 nm and 370 ± 12 nm, respectively.

Adsorption of PEI/DNA complexes onto PLGA nanoparticles was evaluated by zeta potential measurements. As shown in Fig. 2b, zeta potential increased in presence of PEI/DNA complexes (N/P = 20). Zeta potential of blank PLGA nanoparticles was around −25 (±5 mV); after PEI/DNA complexes adsorption and increased to +40 (±3 mV) for 29.8 mg/ml nanoparticle concentration. At the concentration of 1.3 mg/ml of PLGA nanoparticles (lowest nanoparticles concentration), zeta potential was +25 (±7 mV). For 10.9 mg/ml nanoparticles concentration, the zeta potential was already +40 (±3 mV) meaning that a plateau was obtained. This result was confirmed with the following ethidium bromide assay.

### 3.2. Ethidium bromide assay

Analysis of PEI/DNA complex (N/P = 20) efficiency adsorbed onto PLGA nanoparticles was performed with ethidium bromide exclusion assay (Fig. 3). PEI/DNA complexes were adsorbed onto the nanoparticles for different concentrations of PLGA nanoparticles, ranging from 1.3 mg/ml to 29.8 mg/ml. The results showed a significant decrease in percentage of relative fluorescence for all PLGA nanoparticle concentration ($p < 0.05$). It showed that PEI/DNA complexes were efficiently adsorbed onto PLGA nanoparticles starting at 1.3 mg/ml of nanoparticle concentration. These results were consistent with those advanced in measuring the zeta potential.

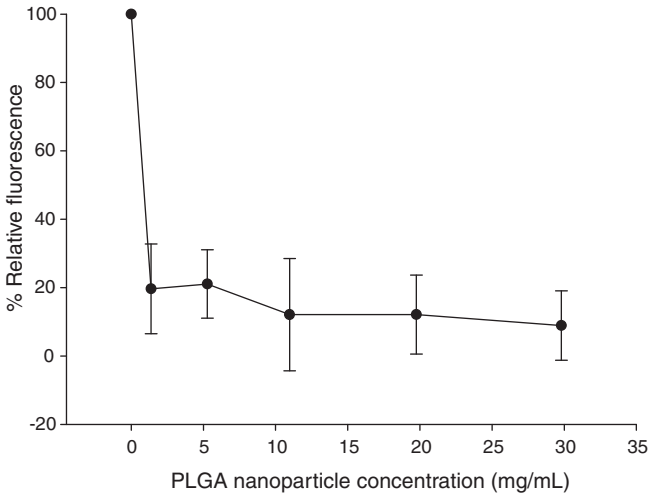


**Fig. 3.** Characterisation of the binding interaction between PLGA nanoparticles and PEI/DNA (N/P = 20) complex. Free PEI/DNA complexes were incubated with ethidium bromide (100% fluorescence), then incubated with PLGA. Data present the means of triplicate samples ± SD.

### 3.3. Agarose gel electrophoresis of pDNA

The agarose gel electrophoresis was carried out to confirm whether the pDNA was associated to the PLGA/PEI nanoparticles (Fig. 4). PLGA/PEI nanoparticles, with or without pDNA, are too large to diffuse through the agarose matrix. Therefore, only pDNA which is not bound onto the surface of nanoparticles is able to migrate to the positive electrode in the same manner as the naked pDNA (Elfinger et al., 2009). Thus, different concentrations of PLGA/PEI nanoparticles were incubated with a constant amount of pDNA in order to determine the optimal N/P ratio. Optimal pDNA adsorption efficiency was achieved for N/P ratios ≥20/1. Contrary, for N/P 1/1, 3/1 and 5/1 ratios, pDNA was not totally bound onto nanoparticles.

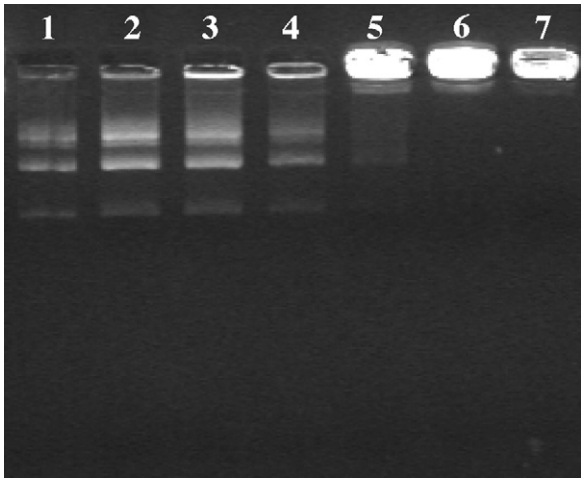


**Fig. 4.** Agarose gel of DNA (4 μg) adsorbed onto different amount of PLGA/PEI nanoparticles with different N/P ratios (NP PLGA/PEI–DNA). The samples from the left to the right were as follows: (lane 1) naked DNA; (lane 2), ratio N/P = 1/1, (lane 3) ratio N/P = 3/1, (lane 4) ratio N/P = 5/1, (lane 5) ratio N/P = 20/1, (lane 6) ratio N/P = 40/1, (lane 7) ratio N/P = 50/1.

### 3.4. In vitro release

An initial increase in release was observed for each type of nanoparticles either with pDNA or PEI/DNA complexes (Fig. 5). The initial release was higher with the nanoparticles adsorbing the PEI/DNA complexes. The initial and overall release for the PLGA–PEI/DNA nanoparticles concentration of 10.9 mg/ml was found to be statistically higher than that observed for the 1.3 and 29.8 mg/ml concentration ($p < 0.05$). The release of PEI/DNA was between 30% and 80% after 24 h for the 29.8 and 10.9 mg/ml nanoparticles concentration, respectively. As for the PLGA/PEI–DNA nanoparticles, the release of pDNA reached almost 25% after 24 h for all N/P ratios. The incorporation of PEI into the PLGA nanoparticles significantly influenced the release rate of pDNA when compared with the nanoparticles adsorption of the PEI/DNA complex ($p < 0.05$).

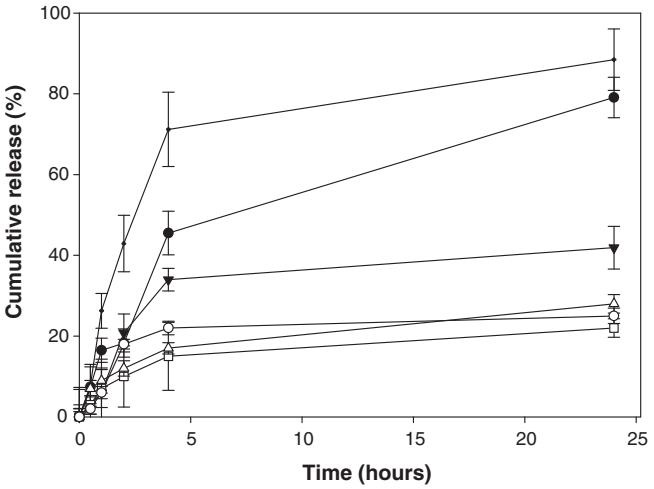


**Fig. 5.** Cumulative release of pDNA and PEI/DNA complex from PLGA/PEI–DNA and PLGA–PEI/DNA nanoparticles into PBS at 37 °C. Data are shown as mean ± SD ($n = 3$). (—●—) PLGA–PEI/DNA 1.3 mg/ml; (—▲—) PLGA–PEI/DNA 10.9 mg/ml; (—▼—) PLGA–PEI/DNA 29.8 mg/ml; (—□—) PLGA/PEI–DNA (N/P = 5); (—△—) PLGA/PEI–DNA (N/P = 10); (—○—) PLGA/PEI–DNA (N/P = 20).

### 3.5. In vitro photochemical transfection and cytotoxicity assay

Gene transfection efficiency was compared between PEI/DNA complexes (N/P ratio from 5 to 20), PLGA/PEI–DNA nanoparticles, and PLGA–PEI/DNA nanoparticles (Fig. 6a–d). In the case of PEI/DNA complexes (Fig. 6b), it was observed that the GFP expression increased when the N/P ratio was increased. However, the differences were not statistically different between the lowest (5) and the highest (20) N/P ratios ($p > 0.05$). Binding the PEI/DNA complexes (only for N/P ratio of 20) onto PLGA nanoparticles, did

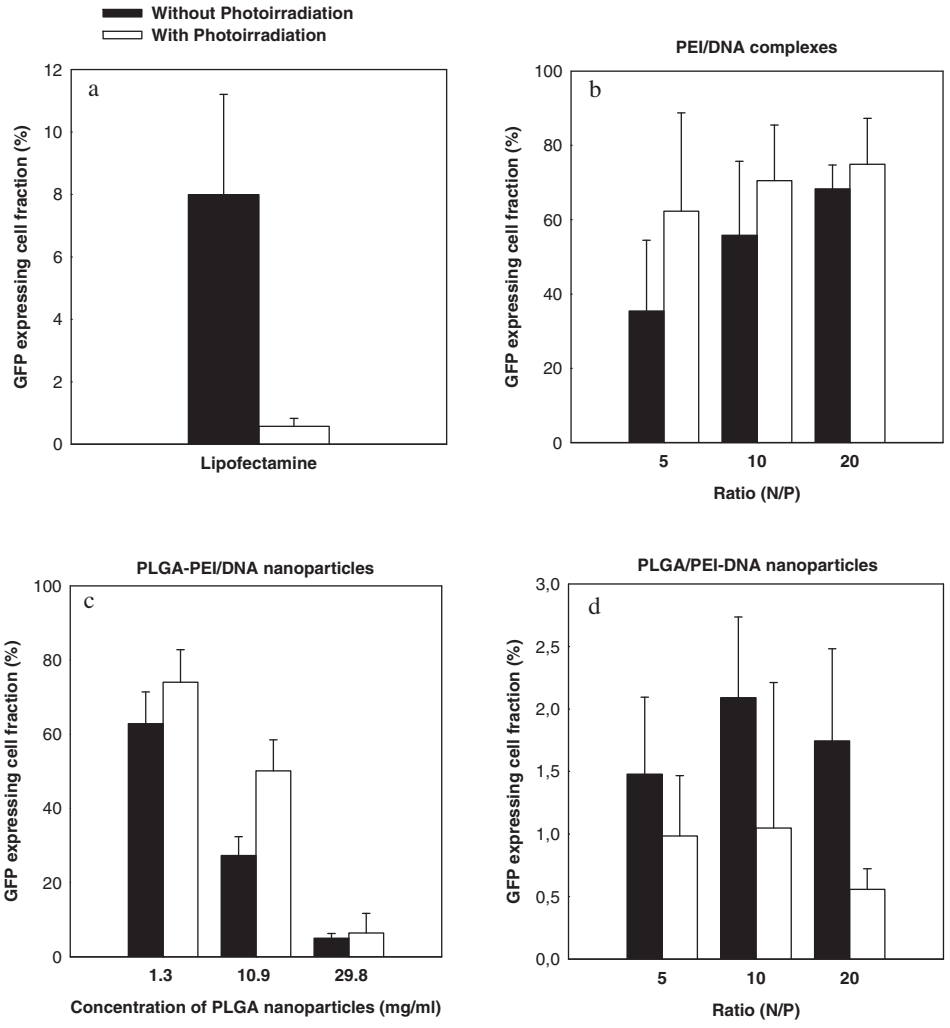


**Fig. 6.** GFP expressing cell fraction (%) in FaDu cells measured by flow cytometry 24 h after GFP gene transfer using (a) Lipofectamine™ 2000, (b) PEI/DNA complex, (c) PLGA–PEI/DNA nanoparticles and (d) PLGA/PEI–DNA nanoparticles with or without photoirradiation ($n = 9 \pm$ SD).

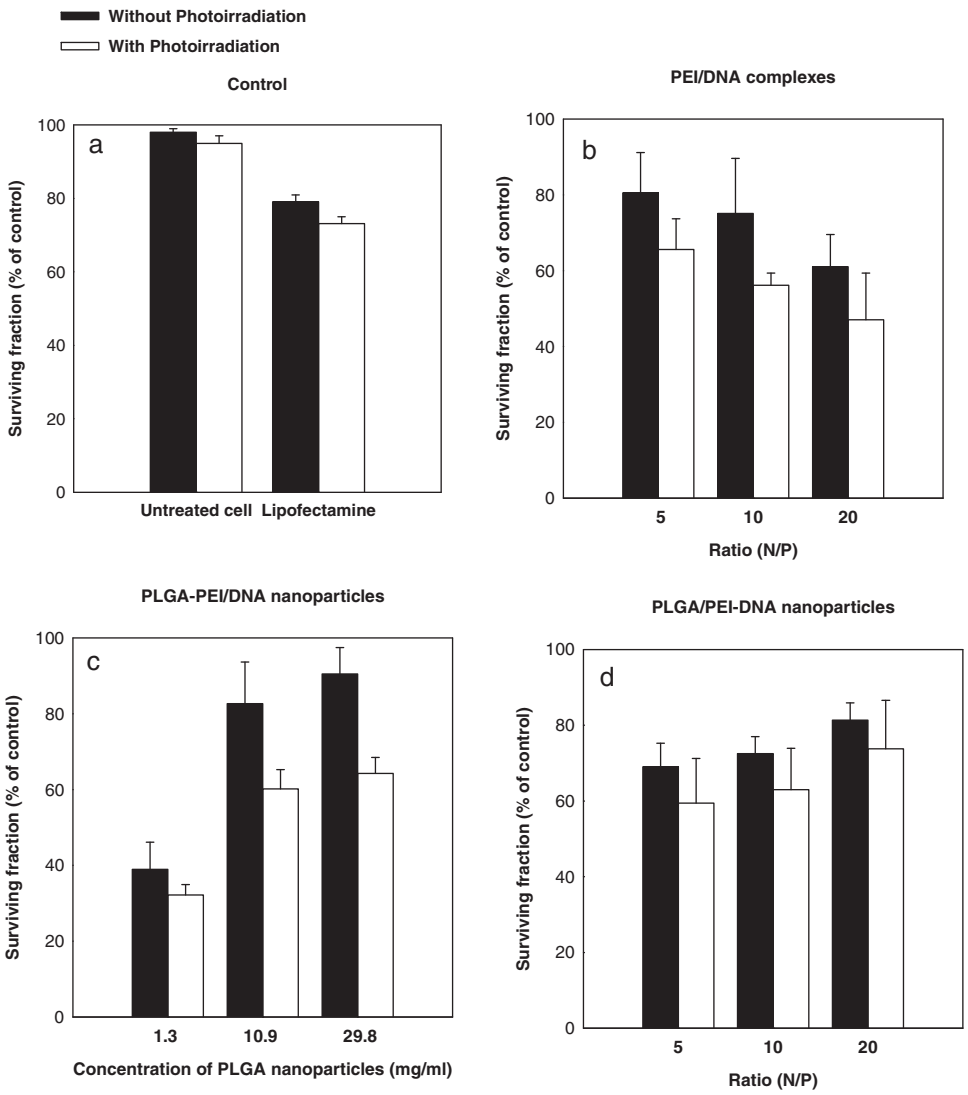


**Fig. 7.** Cytotoxicity of (a) Lipofectamine™ 2000, (b) PEI/DNA complex, (c) PLGA–PEI/DNA nanoparticles and (d) PLGA/PEI–DNA nanoparticles in FaDu cell lines with or without photoirradiation. Results are expressed as the fraction of surviving cells relative to untreated cells without photoirradiation ($n = 9 \pm$ SD).

significantly improve the transfection efficiency ($p < 0.05$, Fig. 6c). On the contrary, the transfection efficiency was decreased for 10.9 and 29.8 mg/ml of nanoparticle concentrations whereas it was similar for 1.3 mg/ml of the concentration. Surprisingly, in the case of pDNA adsorbed onto the PLGA/PEI nanoparticles (Fig. 6d), there was very low transfection efficiency (>3%). When compared with a well-known commercial standard (i.e. Lipofectamine™ 2000), it was observed that the PEI/DNA complexes either alone or bound onto the PLGA nanoparticles (for the 1.3 and 10.9 mg/ml concentration) improved dramatically the transfection efficiency (>60%).

PCI improved the transfection efficiency in PEI/DNA complexes and PLGA–PEI/DNA nanoparticles. However a statistical enhancement of about 2 fold in GFP expression was noticed for the N/P ratio of 5 for the PEI/DNA complexes (Fig. 6b). In the case of the PEI/DNA complexes bound onto PLGA nanoparticles (Fig. 6c), a significant increase was also observed for the 1.3 and 10.9 mg/ml nanoparticle concentrations ($p < 0.05$).

The cytotoxicity of PEI/DNA complexes, PLGA–PEI/DNA and PLGA/PEI–DNA nanoparticles, and Lipofectamine™ 2000 was investigated in FaDu cell line, with or without PCI (Fig. 7a–d). The results revealed that all systems, with or without PCI, were toxic to some extent when compared to untreated cells. Using Lipofectamine™ 2000, approximately 20% loss of cell viability was found, and no significant increase was observed when PCI was used ($p > 0.05$, Fig. 7a). In the case of the PEI/DNA complexes (Fig. 7b), the higher the N/P ratio, the more toxic the system was. When the complex was bound onto the PLGA nanoparticles, toxicity was found to decrease with increasing concentration of nanoparticles (Fig. 7c). PLGA/PEI nanoparticles adsorbing pDNA, toxicity was also observed from 30% to approximately 20% for N/P = 5 and the N/P = 20 ratio, respectively. A slight decrease in toxicity was observed when N/P ratio was increased (Fig. 7d). When PCI was used, cells viability decreased approximately between 10 and 20% for both types of nanoparticles and PEI/DNA complexes.

## 4. Discussion

In recent years, biodegradable nanoparticles, based on PLGA and/or PEI polymers (Bivas-Benita et al., 2004; Kim et al., 2005; Park et al., 2008; Son and Kim, 2010; Takashima et al., 2007) have been developed for gene delivery.

In the present study, the transfection ability and cytotoxicity of PLGA/PEI nanoparticles adsorbing pDNA and PLGA nanoparticles adsorbing PEI/DNA complexes were evaluated. Furthermore, since PCI has been reported to enhance gene delivery (Bonsted et al.,

2008; Nishiyama et al., 2005; Shieh et al., 2008), the impact of PCI onto pDNA delivery from both types of nanoparticles was also evaluated.

Since pDNA is a hydrophilic macromolecule, nanoparticles were prepared by the well-known double emulsion evaporation method (Park et al., 2008). PLGA nanoparticles are neutral or slightly electro-negatively charged particles. PLGA is a biodegradable polymer with two functional end groups i.e. hydroxyl and carboxylic group. The carboxylic functions can interact with the amine groups of PEI via an electrostatic interaction. Since cationic particles are known to improve oligonucleotides delivery, PEI was selected as the cationic polymer designed to be either incorporated or adsorbed in nanoparticles (Katas et al., 2009). However, PEI had several drawbacks: it is generally cytotoxic for cell lines (Neu et al., 2005), the positive charge of PEI/DNA polyplexes engenders interactions with blood components (Rudolph et al., 2002; Zou et al., 2000) and active the complement system which results in rapid clearance of the complex from the bloodstream (Godbey and Mikos, 2001). Thus, the strategy was to use PLGA polymer to decrease cytotoxicity and to avoid these problems.

It is well known that particle size significantly affects the cellular uptake of nanoparticles and, in some cell lines only submicron size particles have been reported to efficient yield in gene transfer (Panyam and Labhasetwar, 2003). The mean diameter of nanoparticles incorporating PEI (PLGA/PEI nanoparticles) depends on the weight ratio of PLGA used during the formulation (Katas et al., 2009). The nanoparticles diameter was slightly decreased when PEI was incorporated into the matrix, and ranged from 380 to 320 nm for PLGA nanoparticles and PLGA/PEI nanoparticles (200 mg/ml of PEI), respectively. In previous study it was observed that the use of PEI in PLGA nanoparticles decreased the particle size with increasing the amount of PEI added (Takashima et al., 2007). When PEI/DNA complex was adsorbed onto the blank PLGA nanoparticles, there was also a slight trend in size reduction since the diameter was around 350 nm. Nevertheless, in both cases, the size difference was not statistically different ($p > 0.05$). Therefore, it was possible to compare transfection and toxicity results with nanoparticles of similar size allowing the size parameter not to influence the observed results.

The negative zeta potential of blank PLGA nanoparticles was attributed to the terminal carboxyl groups of PLGA. For PLGA/PEI nanoparticles, the zeta-potential of PLGA/PEI nanoparticles dramatically changed increasing PEI amounts in the PLGA/PEI formulations. The zeta-potential of the PLGA/PEI nanoparticles (5 mg/ml of PEI) was $+40 \pm 2$ mV. Zeta potential is the result of positive charges present on the surface of nanoparticles. Since the zeta potential is stable at +40 mV, it can be concluded that the same amount of positive charges is present on the nanoparticles surface. So two hypotheses can be dawn: (1) PEI molecules are released in the outer aqueous phase meaning that the particles are already saturated with the lowest PEI concentration. (2) Major part of PEI is distributed in the matrix of the particles and do not have any action on the zeta potential since charge are hidden inside the nanoparticles. In the literature, similar results were obtained for PLGA–PEI nanoparticles using PVA (5%, w/v). The particle surface charge was $+37.4 \pm 1.0$ mV (Katas et al., 2009). The positive charge was a result of the adsorption of PEI onto the surface of nanoparticles. The positively charged particle surface can facilitate adherence to negatively charged cellular membranes, thus inducing and increasing intracellular uptake (Mansouri et al., 2006).

In the case of PLGA–PEI/DNA nanoparticles, a constant amount of PEI/DNA complex (N/P = 20) was added. For the lowest concentration of PLGA nanoparticles (1.3 mg/ml), zeta potential was around $+25 \pm 7$ mV. However, a constant value of around $+40 \pm 3$ mV was obtained for 5.0–29.8 mg/ml of PLGA nanoparticles concentrations.

In the PLGA–PEI/DNA complex nanoparticle solution, the zeta potential was positive and comparable to that of PEI/DNA due to possible attachment of PEI/DNA particles on the surface of PLGA nanoparticles (Chumakova et al., 2008).

EtBr assay was used to evaluate the zeta potential of PEI/DNA complex onto PLGA nanoparticles. According to EtBr assay, when PLGA nanoparticle concentration increased the percentage of the relative fluorescence decreased. This result might be explained due to the PEI/DNA complexes adsorption onto the PLGA nanoparticles. Incubation of plasmid with polylysine gave similar results by the reduction EtBr fluorescence (Chaszczewska-Markowska et al., 2004). For both type of nanoparticles, the system was very rapidly saturated with PEI/DNA complexes and the zeta potential reached the same value i.e. around $+40 \pm 2$ mV.

For PLGA/PEI nanoparticles, pDNA adsorption was determined by an agarose gel technique. For a 20/1 ratio, pDNA is totally adsorbed onto PLGA/PEI nanoparticles. This result suggests that the negatively charged pDNA gradually interacted with the cationic surface of PLGA/PEI nanoparticles up to this ratio. Following this ratio, the charge interactions achieved saturation conditions.

The release kinetic of the nanoparticles formulations was performed in a PBS buffer (pH 7.4). Indeed, the release kinetics and the transfection efficiency of pDNA might be influenced by the presence of PEI in the PLGA/PEI–DNA nanoparticles. In the case of PLGA–PEI/DNA nanoparticles, more than 35% of PEI/DNA complex was released within 4 h, which means that transfection was due to the PEI/DNA complexes. In this case, it can be considered that nanoparticles target PEI/DNA complex to cells. For the PLGA/PEI–DNA nanoparticles, not more than 25% of pDNA was released after 4 h and for up to 24 h. This slow release may explain the lower transfection results observed with this type of nanoparticles. This result can be explained (i) by the cationic nature of PEI that may retard pDNA release due to the electrostatic attractions between them (Son and Kim, 2010) or (ii) by the type of nanoparticles and the efficiency of the pDNA complexation with PEI.

These results are somehow contradictory between the gel retardation assay and the *in vitro* release test but could be explained. In the gel retardation assay, nanoparticles are sampled in a well and submitted to an electrophoretic field. There is no dilution of the nanoparticles and only the free pDNA can move showing that there was free pDNA for the 5 and 10 N/P ratios but not for the 20 N/P ratio. The situation is totally different in an in-vitro release test. Indeed, nanoparticles were diluted with PBS (200 μl of nanoparticles in 200 μl of PBS buffer so a dilution of 1/2). Dilution is always responsible for "drug" release unless the "drug" is covalently bound, which was not the case in our experiment. This explains why the 20 N/P ratio nanoparticles were still able to transfect (although poorly) the FADU cells. In addition, there were no statistical differences in the transfection efficiency of the 3 N/P ratios. As a conclusion, the gel retardation assay and the *in vitro* dissolution test do not show the same thing and the dissolution test reflect more the possibility for the pDNA to be at least partially released and therefore display a transfection efficacy.

Transfection efficiency and cytotoxicity have been shown to be depending on the N/P ratios (PEI/DNA) (Godbey et al., 1999), and the photochemical internalization (Berg et al., 1999). In our case, we have selected 3 ratios (N/P = 5, 10 or 20) where pDNA was totally complexed with PEI (data not shown).

For PLGA/PEI–DNA nanoparticles, the transfection efficiency was approximately 3%. The low transfection efficiency could be attributed to a low nanoparticles endocytosis from the nanoparticles. Similarly, Kim et al. (2005) evaluated β-galactosidase transfection with the same type of PLGA/PEI nanoparticles and obtained transfection results of approximately 10%. Bivas-Benita et al. (2004) also reported that PLGA/PEI–DNA nanoparticles were

taken up in Calu-3 cells. However, pDNA encoding for the GFP protein expression signal was rather low.

For PLGA–PEI/DNA nanoparticles, transfection efficiency were close to 70% for 1.3 mg/ml of PLGA nanoparticles as observed for the PEI/DNA complexes (N/P = 20). From the kinetic release result, we hypothesize that only PEI/DNA complexes are internalized in cells.

Cytotoxicity is also a critical issue that may affect transfection efficiency (Choi et al., 2006). Both types of nanoparticles were slightly less toxic than free PEI/DNA complexes: this result was more obvious for the N/P = 20 ratio. Furthermore, higher PEI/DNA complexes ratio correlates with higher cytotoxicity. It was previously reported that cell toxicity can be reduced by using lower PEI/DNA ratio (Florea et al., 2002). In addition, when PEI was incorporated in the PLGA nanoparticles, the toxicity of PEI was decreased. In the literature, the effect of PEI and siRNA incorporation in PLGA nanoparticles on cytotoxicity was evaluated (Patil and Panyam, 2009): nanoparticle formulations were found to be non-toxic to the cells whereas free PEI was found to be highly toxic.

In this research, we also evaluated the combination of both type of nanoparticles with PCI on the transfection efficiency and the cytotoxicity. For PEI/DNA complexes and PLGA–PEI/DNA nanoparticles, PCI increase the efficiency of transfection. The mechanism of PCI is based on photochemical rupture of endocytotic vesicles after incubation of the cells with a photosensitizing compound which localizes to these vesicles, and irradiation at a wavelength specific to the photosensitizer (Ndoye et al., 2004b). We can hypothesize that PLGA nanoparticles can stimulate the binding of PEI/DNA to the cell membrane and the endocytosis. The toxicity of nanoparticles was increased by direct interaction with PCI. Cytotoxicity of the photosensitizer may be ruled out since the cell viability is almost 100% with TPPS2a. From our experimental results, it can be concluded that PCI is more toxic than nanoparticles since, when applied together, there is an important increase of cells death. Nevertheless, the details of the interaction of PCI with the nanoparticles are still unclear.

In our case, GFP expression was found to be higher when PCI was applied with PEI/DNA complexes. For PLGA–PEI/DNA nanoparticles, PCI improved significantly the transfection efficiency for the 1.3 and 10.9 mg/ml nanoparticle concentrations ($p < 0.05$). These findings suggest that the combination of the nanoparticle with PCI is an efficient tool for gene delivery. However, for the PLGA/PEI–DNA nanoparticles the transfection efficiency decreased with PCI. These results might be explained as follows: PCI damage can explain the decrease in transfection seen after PCI with some cationic lipid transfection agents. This indicates that photochemical damage may be more important for some transfection agents than for others, either because some complexes are located closer to the sensitiser, or because different complexing agents protect the DNA from damage to a different extent. The impact of PCI onto the two systems was totally different. First, the PCI efficiency depends on the photosensitizer, cells lines, and molecule endocytosis. Second, in order to enhance transfection efficiency with PCI, it is necessary that transfection works at least a minimum without PCI in order to obtain a synergistic effect. The importance of photochemical damage for the overall efficiency of PCI-mediated drug delivery is not known and will probably vary substantially with the molecule to be internalized, and may be also with the photosensitiser used (Hogset et al., 2004).

## 5. Conclusion

From this research, PEI has been shown to have the ability to deliver pDNA into cells. Modification of PLGA nanoparticles by incorporating PEI or adsorbing PEI/DNA complexes onto nanoparticles has led the formation of a delivery system for pDNA. Cytotoxicity of nanoparticles and complexes were investigated with MTT test, and the result showed that PCI increased by approximately 20% the cytotoxicity of nanoparticles and the complexes. PCI was found to slightly improve the transfection efficiency for all formulations.

## References


Berg, K., Dietze, A., Kaalhus, O., Hogset, A., 2005. Site-specific drug delivery by photochemical internalization enhances the antitumor effect of bleomycin. Clin. Cancer Res. 11, 8476–8485.

Berg, K., Selbo, P.K., Prasmickaite, L., Tjelle, T.E., Sandvig, K., Moan, J., Gaudernack, G., Fodstad, O., Kjolsrud, S., Anholt, H., Rodal, G.H., Rodal, S.K., Hogset, A., 1999. Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. Cancer Res. 59, 1180–1183.

Bivas-Benita, M., Romeijn, S., Junginger, H.E., Borchard, G., 2004. PLGA–PEI nanoparticles for gene delivery to pulmonary epithelium. Eur. J. Pharm. Biopharm. 58, 1–6.

Bonsted, A., Engesaeter, B.O., Hogset, A., Maelandsmo, G.M., Prasmickaite, L., Kaalhus, O., Berg, K., 2004. Transgene expression is increased by photochemically mediated transduction of polycation-complexed adenoviruses. Gene Ther. 11, 152–160.

Bonsted, A., Wagner, E., Prasmickaite, L., Hogset, A., Berg, K., 2008. Photochemical enhancement of DNA delivery by EGF receptor targeted polyplexes. Methods Mol. Biol. 434, 171–181.

Budker, V., Zhang, G., Knechtle, S., Wolff, J.A., 1996. Naked DNA delivered intraportally expresses efficiently in hepatocytes. Gene Ther. 3, 593–598.

Chaszczewska-Markowska, M., Ugorski, M., Langner, M., 2004. Plasmid condensation induced by cationic compounds: hydrophilic polylysine and amphiphilic cationic lipid. Cell. Mol. Biol. Lett. 9, 3–13.

Choi, J.S., Ko, K.S., Park, J.S., Kim, Y.H., Kim, S.W., Lee, M., 2006. Dexamethasone conjugated poly(amidoamine) dendrimer as a gene carrier for efficient nuclear translocation. Int. J. Pharm. 320, 171–178.

Chumakova, O.V., Liopo, A.V., Andreev, V.G., Cicenaite, I., Evers, B.M., Chakrabarty, S., Pappas, T.C., Esenaliev, R.O., 2008. Composition of PLGA and PEI/DNA nanoparticles improves ultrasound-mediated gene delivery in solid tumors in vivo. Cancer Lett. 261, 215–225.

Cohen, H., Levy, R.J., Gao, J., Fishbein, I., Kousaev, V., Sosnowski, S., Slomkowski, S., Golomb, G., 2000. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. Gene Ther. 7, 1896–1905.

Dietze, A., Bonsted, A., Hogset, A., Berg, K., 2003. Photochemical internalization enhances the cytotoxic effect of the protein toxin gelonin and transgene expression in sarcoma cells. Photochem. Photobiol. 78, 283–289.

Elfinger, M., Pfeifer, C., Uezguen, S., Golas, M.M., Sander, B., Maucksch, C., Stark, H., Aneja, M.K., Rudolph, C., 2009. Self-assembly of ternary insulin-polyethylenimine (PEI)-DNA nanoparticles for enhanced gene delivery and expression in alveolar epithelial cells. Biomacromolecules 10, 2912–2920.

Fay, F., Quinn, D.J., Gilmore, B.F., McCarron, P.A., Scott, C.J., 2010. Gene delivery using dimethyldidodecylammonium bromide-coated PLGA nanoparticles. Biomaterials 31, 4214–4222.

Florea, B.I., Meaney, C., Junginger, H.E., Borchard, G., 2002. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. AAPS PharmSci 4, E12.

Folini, M., Berg, K., Millo, E., Villa, R., Prasmickaite, L., Daidone, M.G., Benatti, U., Zaffaroni, N., 2003. Photochemical internalization of a peptide nucleic acid targeting the catalytic subunit of human telomerase. Cancer Res. 63, 3490–3494.

Godbey, W.T., Mikos, A.G., 2001. Recent progress in gene delivery using non-viral transfer complexes. J. Control. Release 72, 115–125.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J. Biomed. Mater. Res. 45, 268–275.

Ho, J., Wang, H., Forde, G.M., 2008. Process considerations related to the microencapsulation of plasmid DNA via ultrasonic atomization. Biotechnol. Bioeng. 101, 172–181.

Hogset, A., Prasmickaite, L., Selbo, P.K., Hellum, M., Engesaeter, B.O., Bonsted, A., Berg, K., 2004. Photochemical internalisation in drug and gene delivery. Adv. Drug Deliv. Rev. 56, 95–115.

Katas, H., Cevher, E., Alpar, H.O., 2009. Preparation of polyethyleneimine incorporated poly(D,L-lactide-co-glycolide) nanoparticles by spontaneous emulsion diffusion method for small interfering RNA delivery. Int. J. Pharm. 369, 144–154.

Kim, I.S., Lee, S.K., Park, Y.M., Lee, Y.B., Shin, S.C., Lee, K.C., Oh, I.J., 2005. Physicochemical characterization of poly(L-lactic acid) and poly(D,L-lactide-co-glycolide) nanoparticles with polyethylenimine as gene delivery carrier. Int. J. Pharm. 298, 255–262.

Kim, W.J., Chang, C.W., Lee, M., Kim, S.W., 2007. Efficient siRNA delivery using water soluble lipopolymer for anti-angiogenic gene therapy. J. Control. Release 118, 357–363.

Kumar, M.N., Mohapatra, S.S., Kong, X., Jena, P.K., Bakowsky, U., Lehr, C.M., 2004. Cationic poly(lactide-co-glycolide) nanoparticles as efficient in vivo gene transfection agents. J. Nanosci. Nanotechnol. 4, 990–994.

Liu, L., Xu, H.M., Jiang, H., Wang, J., Song, N., Xie, J.X., 2010. Recombinant adenovirus-mediated expression of GHS-R1a in HEK 293 cells. Neurosci. Bull. 26, 225–231.

Mansouri, S., Cuie, Y., Winnik, F., Shi, Q., Lavigne, P., Benderdour, M., Beaumont, E., Fernandes, J.C., 2006. Characterization of folate–chitosan–DNA nanoparticles for gene therapy. Biomaterials 27, 2060–2065.

Ndoye, A., Bouali, S., Dolivet, G., Leroux, A., Erbacher, P., Behr, J.P., Berg, K., Guillemin, F., Merlin, J.L., 2004a. Sustained gene transfer and enhanced cell death following glucosylated-PEI-mediated p53 gene transfer with photochemical internalisation in p53-mutated head and neck carcinoma cells. Int. J. Oncol. 25, 1575–1581.

Ndoye, A., Dolivet, G., Hogset, A., Leroux, A., Fifre, A., Erbacher, P., Berg, K., Behr, J.P., Guillemin, F., Merlin, J.L., 2006. Eradication of p53-mutated head and neck squamous cell carcinoma xenografts using nonviral p53 gene therapy and photochemical internalization. Mol. Ther. 13, 1156–1162.

Ndoye, A., Merlin, J.L., Leroux, A., Dolivet, G., Erbacher, P., Behr, J.P., Berg, K., Guillemin, F., 2004b. Enhanced gene transfer and cell death following p53 gene transfer using photochemical internalisation of glucosylated PEI–DNA complexes. J. Gene Med. 6, 884–894.

Neu, M., Fischer, D., Kissel, T., 2005. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J. Gene Med. 7, 992–1009.

Nishiyama, N., Iriyama, A., Jang, W.D., Miyata, K., Itaka, K., Inoue, Y., Takahashi, H., Yanagi, Y., Tamaki, Y., Koyama, H., Kataoka, K., 2005. Light-induced gene transfer from packaged DNA enveloped in a dendrimeric photosensitizer. Nat. Mater. 4, 934–941.

Niu, X., Zou, W., Liu, C., Zhang, N., Fu, C., 2009. Modified nanoprecipitation method to fabricate DNA-loaded PLGA nanoparticles. Drug Dev. Ind. Pharm. 35, 1375–1383.

Niven, R., Zhang, Y., Smith, J., 1997. Toward development of a non-viral gene therapeutic. Adv. Drug Deliv. Rev. 26, 135–150.

O'Rorke, S., Keeney, M., Pandit, A., 2010. Non-viral polyplexes: scaffold mediated delivery for gene therapy. Prog. Polym. Sci. 35, 441–458.

Oliveira, A.C., Ferraz, M.P., Monteiro, F.J., Simoes, S., 2009. Cationic liposome–DNA complexes as gene delivery vectors: development and behaviour towards bone-like cells. Acta Biomater. 5, 2142–2151.

Panyam, J., Labhasetwar, V., 2003. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv. Drug Deliv. Rev. 55, 329–347.

Panyam, J., Zhou, W.Z., Prabha, S., Sahoo, S.K., Labhasetwar, V., 2002. Rapid endo-lysosomal escape of poly(D,L-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J. 16, 1217–1226.

Park, Y.M., Shin, B.A., Oh, I.J., 2008. Poly(L-lactic acid)/polyethylenimine nanoparticles as plasmid DNA carriers. Arch. Pharm. Res. 31, 96–102.

Patil, Y., Panyam, J., 2009. Polymeric nanoparticles for siRNA delivery and gene silencing. Int. J. Pharm. 367, 195–203.

Rhaese, S., von Briesen, H., Rubsamen-Waigmann, H., Kreuter, J., Langer, K., 2003. Human serum albumin–polyethylenimine nanoparticles for gene delivery. J. Control. Release 92, 199–208.

Rudolph, C., Schillinger, U., Plank, C., Gessner, A., Nicklaus, P., Müller, R.H., Rosenecker, J., 2002. Nonviral gene delivery to the lung with copolymer-protected and transferrin-modified polyethylenimine. Biochim. Biophys. Acta-Gen. Subjects 1573, 75–83.

Sahoo, S.K., Panyam, J., Prabha, S., Labhasetwar, V., 2002. Residual polyvinyl alcohol associated with poly(D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J. Control. Release 82, 105–114.

Shieh, M.J., Peng, C.L., Lou, P.J., Chiu, C.H., Tsai, T.Y., Hsu, C.Y., Yeh, C.Y., Lai, P.S., 2008. Non-toxic phototriggered gene transfection by PAMAM–porphyrin conjugates. J. Control. Release 129, 200–206.

Singh, M., Briones, M., Ott, G., O'Hagan, D., 2000. Cationic microparticles: a potent delivery system for DNA vaccines. Proc. Natl. Acad. Sci. U.S.A. 97, 811–816.

Son, S., Kim, W.J., 2010. Biodegradable nanoparticles modified by branched polyethylenimine for plasmid DNA delivery. Biomaterials 31, 133–143.

Takashima, Y., Saito, R., Nakajima, A., Oda, M., Kimura, A., Kanazawa, T., Okada, H., 2007. Spray-drying preparation of microparticles containing cationic PLGA nanospheres as gene carriers for avoiding aggregation of nanospheres. Int. J. Pharm. 343, 262–269.

Wang, K., Cheng, L., Liang, Y., Liu, D., Li, K., Wang, P., 2010. Adenovirus-mediated delivery of shRNA against bFGF mRNA suppresses growth of cultured human primary prostatic stromal cells. Mol. Biol. Rep., doi:10.1007/s11033-010-0191-2.

Wolfe, D., Craft, A.M., Cohen, J.B., Glorioso, J.C., 2010. An HSV Vector System for Expression of Complex Cellular cDNA Libraries. J. Virol. 84, 7360–7368.

Xiao, W., Chen, X., Yang, L., Mao, Y., Wei, Y., Chen, L., 2010. Co-delivery of doxorubicin and plasmid by a novel FGFR-mediated cationic liposome. Int. J. Pharm. 393, 119–126.

Yang, S.J., Chang, S.M., Tsai, K.C., Chen, W.S., Lin, F.H., Shieh, M.J., 2010. Effect of chitosan-alginate nanoparticles and ultrasound on the efficiency of gene transfection of human cancer cells. J. Gene Med. 12, 168–179.

Zou, S.M., Erbacher, P., Remy, J.S., Behr, J.P., 2000. Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. J. Gene Med. 2, 128–134.