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Research paper

PLGA–PEI nanoparticles for gene delivery to pulmonary epithelium

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Abstract

Pulmonary gene delivery is thought to play an important role in treating genetically related diseases and may induce immunity towards pathogens entering the body via the airways. In this study we prepared poly (D,L-lactide-*co*-glycolide) (PLGA) nanoparticles bearing polyethyleneimine (PEI) on their surface and characterized them for their potential in serving as non-viral gene carriers to the pulmonary epithelium. Particles that were synthesized at different PLGA–PEI ratios and loaded with DNA in several PEI–DNA ratios, exhibited narrow size distribution in all formulations, with mean particle sizes ranging between 207 and 231 nm. Zeta potential was strongly positive (above 30 mV) for all the PEI–DNA ratios examined and the loading efficiency exceeded 99% for all formulations. Internalization of the DNA-loaded PLGA–PEI nanoparticles was studied in the human airway submucosal epithelial cell line, Calu-3, and DNA was detected in the endo-lysosomal compartment 6 h after particles were applied. Cytotoxicity of these nanoparticles was dependent on the PEI–DNA ratio and best cell viability was achieved by PEI–DNA ratios 1:1 and 0.5:1. These findings demonstrate that PLGA–PEI nanoparticles are a potential new delivery system to carry genes to the lung epithelium.

Keywords: Poly(D,L-lactide-*co*-glycolide) nanoparticles; Polyethyleneimine; Calu-3 cells; Non-viral gene delivery; Pulmonary delivery

1. Introduction

Pulmonary gene delivery and DNA vaccination are of considerable interest due to the variety of lung diseases that can be addressed by this approach. Diseases like cystic fibrosis, asthma, chronic obstructive pulmonary disease, lung cancer and more could be treated by high-level and long-term expression of the corresponding gene of interest [1–5]. Furthermore, many pathogens that enter the body through the airways, causing pulmonary as well as systemic diseases like *Mycobacterium tuberculosis*, influenza and SARS-associated coronavirus (SARS–CoV) could be controlled once appropriate vaccination strategies would be applied [6–9]. Pulmonary vaccination against air-borne pathogens holds the promise of inducing both systemic and local immune responses, resulting in the induction of IgA mucosal antibodies that can prevent the pathogen's entry at the level of the mucosal surface [10,11].

Gene delivery in humans requires carriers that will transfer DNA into the nuclei of target cells. These carriers must be efficient in transfection, safe for human use, protect the DNA from degradation before arriving at the target cell and possibly hold targeting qualities for the specific delivery of the gene to the required cells or tissue. The two approaches for gene delivery are viral and non-viral systems. Although very efficient in transfection, viral gene delivery systems still face safety issues for human use and due to their inherent immunogenicity, are problematic when repeated doses with the same carrier are required. Non-viral delivery systems are generally synthetic agents that are much safer, however, their transfection efficiency in-vivo is limited [12,13].

Much attention was given to the biodegradable and biocompatible poly (D,L-lactide-*co*-glycolide) (PLGA) polymers. These polymers were extensively evaluated for their ability to deliver a variety of therapeutic agents by targeted and/or sustained delivery, were tested for toxicity and safety in animal models and are currently used in humans for resorbable sutures, bone implants and scaffolds in tissue engineering [14–16]. PLGA nanoparticles were shown to escape from the endo-lysosomal compartment to the cytoplasmic compartment and release their contents over extended periods of time [17,18]. These properties rendered PLGA nanoparticles as potential efficient

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sustained release gene delivery systems. Furthermore, PLGA particles were shown to be taken up by antigen presenting cells (APCs) like macrophages and dendritic cells (DCs), therefore proving their ability to serve as DNA vaccine carriers [19,20]. In cases where adsorption of DNA on the surface was desired, positively charged agents like cetyltrimethylammonium bromide (CTAB) were used to produce PLG particles that are positively charged at physiological pH. These particles were able to induce higher antibody and cytotoxic immune responses to HIV-1 pCMV p55 gag plasmid in vivo [21–23].

In order to achieve efficient pulmonary gene expression, the addition of the cationic polymer, polyethyleneimine (PEI), to the PLGA nanoparticles was explored. PEIs exhibit a high positive charge density when protonated in aqueous solutions and are considered to be promising candidates as non-viral vectors for delivery of DNA and oligonucleotides, for in-vitro and in-vivo applications [24,25]. Branched PEI was reported to be associated with successful pulmonary gene delivery, initiated in-vivo transfection and induced only low levels of cytokine production in the lungs [26–29].

In this study, new PLGA particles with PEI attached to their surface were prepared and characterized with regard to size, zeta-potential and loading efficiency. The intracellular pathway of these particles was evaluated together with their cytotoxicity in the human bronchial cell line Calu-3 in order to assess their potential in serving as pulmonary gene delivery system.

2. Materials and methods

2.1. Materials

D, L-Lactide/Glycolide copolymer (PLGA, PURASORB®, DL663FL, molar ratio: 53/47, inherent viscosity 0.69 dl/g) was a generous gift from PURAC (Gorinchem, The Netherlands). Twenty-five kD branched water-free polyethyleneimine (PEI), Tween-80®, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), penicillin and streptomycin were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Poloxamer 188 (Lutrol F68) was purchased from BASF (Ludwigshafen, Germany). Acetone of analytical grade was from Fisher chemicals (Leicestershire, UK) and dichloromethane of analytical grade from Biosolve BV (Valkenswaard, The Netherlands). Ninety-six flat buttom well-plates and 12-wells Transwells® were obtained from Corning (Schiphol, The Netherlands). Dulbecco's Modified Eagle Medium (DMEM) and Hank's Balanced Salt Solution (HBSS) were purchased from Gibco (Basel, Switzerland).

2.2. Preparation of PLGA–PEI nanoparticles

The preparation method of PLGA–PEI nanoparticles was a modification of the technique described by Wehrle et al. [30]. A solution of 10% (w/v) PLGA in Dichloromethane was stirred for 30 min. PEI solution in acetone was prepared to a final concentration of 1% (w/v). PEI was added to the PLGA solution according to the requested PLGA–PEI ratio (we investigated PLGA–PEI w/w ratios of 25:1 and 10:1), Tween-80® added to a final concentration of 1% (w/v) and acetone added up to 10 ml. This organic phase was mixed and poured into an aqueous phase of 20 ml of 0.5% (w/v) Poloxamer-188 in milli-Q®, under moderate stirring at room temperature. Since the acetone diffused into the aqueous phase rapidly, particles formation was observed immediately by the milky color of the aqueous phase. The dichloromethane was rotor evaporated under reduced pressure and then the suspension was filtered through 4–7 μm retention size paper filter ($595\frac{1}{2}$) folded filters, Schleicher and Schuell, s'Hertogenbosch, The Netherlands) to remove large polymer particles. This suspension was then used for the loading of DNA.

2.3. Loading PLGA–PEI nanoparticles with plasmid DNA

PLGA–PEI nanoparticles were loaded withV1Jns DNA plasmid or V1Jns encoding Antigen 85B of *Mycobacterium tuberculosis* (a gift from the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center) as a model for tuberculosis DNA vaccine in the characterization studies. PLGA–PEI nanoparticles suspension was added to a solution of 25 μg/ml DNA plasmid and vortexed for 30 s. The amount of PLGA–PEI nanoparticles suspension added changed according to the PEI–DNA ratio requested (ranging from 0.5:1 up to 5:1). Formulations were left at room temperature for at least 1 h before use.

2.4. Particle size and Zeta potential measurements

Particle size and zeta potential measurements were performed using Zetasizer® 3000 HSA (Malvern Instruments, Bergen op Zoom, The Netherlands). Particle size was measured by photon correlation spectroscopy (PCS) at 25 °C and a fixed 90° scattering angle. Zeta potential determinations were based on electrophoretic mobility of the nanoparticles in the aqueous medium.

2.5. Morphological characterization of nanoparticles by scanning electron microscopy

Polycarbonate membrane filters (Nucleopore®, 13 mm filters, 0.1 μm pore size; Whatman, Kent, UK) were placed on a humidified paper filter. Samples were applied on the filters and left to dry overnight at room temperature. The loaded filters were coated with gold to a thickness of 6 nm using Emitech K650X large sample coater (Emitech, Kent, UK). Samples were visualized using Jeol JSM-6700F field emission scanning electron microscope (JEOL BV, Schiphol-Rijk, The Netherlands).

2.6. Loading efficiency of PLGA–PEI nanoparticles

The amount of encapsulated DNA in the nanoparticles was calculated by measuring the difference between the amount of DNA added to the nanoparticles solution and the measured non-entrapped DNA remaining in the aqueous phase after nanoparticle formation. After formation, the nanoparticle suspension was centrifuged for 15 min at 14,000 rpm and the supernatant was checked for the non-bound DNA concentration with PicoGreen® ds DNA quantitation assay (Molecular Probes, Leiden, The Netherlands) using a Perkin Elmer 7000 Fluorescence Bio assay reader.

2.7. Calu-3 cell line

Calu-3 cells were grown in DMEM supplemented with 10% FCS and 50 μg/ml penicillin and streptomycin at 37 °C in an incubator with 90% humidity and 5% CO_2. A week later, cells were trypsinized and seeded in 4×10^5 cells/ml seeding density in 12-well Transwells® for intracellular staining experiments or in 96-well plates for cytotoxicity studies. Experiments were performed with cells of passage numbers 38–48.

2.8. Visualization of intracellular localization of nanoparticles loaded with plasmid DNA in Calu-3 cells and intracellular protein expression using confocal laser scanning microscopy (CLSM)

For the intracellular localization studies, the procedure of Denis-Mize et al. [22] was used with slight modifications. To evaluate the internalization of the DNA loaded PLGA–PEI nanoparticles and their intracellular localization we used the air-interface culture conditions for Calu-3 cells where the apical culture medium is removed after 1 day of culture. Seven days after seeding, rhodamine-labeled plasmid DNA with hCMV IE promoter/enhancer driving green fluorescent protein gene (pGeneGrip™ Rhodamine/GFP, Gene Therapy Systems, San Diego, CA, USA) bound to PLGA–PEI nanoparticles suspended in HBSS–HEPES, pH 7.4 was added to the wells at 1 μg DNA/well (PLGA–PEI ratio of 10:1; PEI–DNA ratio of 2:1). Cells were incubated with formulations for 4 h and then washed twice and the incubation continued at air-interface. For the co-localization studies, cells were washed, fixated and permeabilized using the Cytofix/Cytoperm™ kit according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium) and stained with mouse anti-human monoclonal antibody for the lysosomal associated membrane protein, LAMP-1 (clone H4A3, BD Pharmingen, Erembodegem, Belgium) followed by incubation with Alexa Fluor® 633 goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands). Cells were washed, applied to glass microscope slides and visualized using Bio-Rad 2100MP (multi-photon) confocal microscope (equipped with Argon 488 nm, HeliumNeon 543 nm and Red diode 637 nm lasers; Veenendaal, The Netherlands) with inverted TE 2000U Nikon microscope (Uvikon, Bunnik, The Netherlands). To detect GFP protein expression 48 h after the formulations were applied, the filters were cut out of the Transwell® insert, placed on a glass slide, covered with cover glass and immediately visualized under the confocal microscope.

2.9. Cytotoxicity of PLGA–PEI nanoparticles

Two days after cells were seeded into the 96 well plates, cells were washed and formulations in HBSS–HEPES, pH 7.4 were applied on cells at a concentration of 1 μg DNA/well. Cell culture medium was used as a positive control and 0.1 mg/ml NaOH/10 mg/ml SDS solution as negative control for cell viability. After 4 h, cells were washed twice and CellTiter 96® aqueous one solution cell proliferation assay (Promega, Leiden, The Netherlands) was performed according to the manufacturer's instructions. Absorbance was measured at 490 nm using a BioTek EL × 808 microplate reader (Winooski, Vt, USA).

3. Results and discussion

3.1. Particle size, Zeta potential and morphology of PLGA–PEI nanoparticles

The particle size of the nanoparticles was between 207 and 231 nm for all PLGA–PEI ratios and PEI–DNA ratios examined (Table 1). The size measurements revealed a polydispersity index lower than 0.2, indicating narrow particle size distributions. The stable particle size and the narrow distribution suggest that the preparation technique is reproducible and results in the formation of uniform particles at both PLGA–PEI ratios. DNA loading did not change particle size for PEI–DNA ratios between 5:1 and 0.5:1. Although particles in the micrometer size range have better lung deposition than nanoparticles [31], it was shown that nanoparticles have relatively higher intracellular uptake compared to microparticles and that in some cell-lines, only

Table 1
Size and Zeta (ζ) potential of PLGA–PEI nanoparticles with different PLGA–PEI ratios and PEI–DNA ratios

PEI–DNA ratio	PLGA–PEI ratio 25:1		PLGA–PEI ratio 10:1	
	Size (nm)	ζ potential (mV)	Size (nm)	ζ potential (mV)
Not loaded	211 ± 8	51.1 ± 5.6	231 ± 12	58.8 ± 4.0
5:1	207 ± 11	52.3 ± 8.3	224 ± 10	53.5 ± 4.7
3:1	209 ± 10	48.5 ± 2.6	221 ± 7	53.5 ± 4.0
2:1	211 ± 9	46.9 ± 3.7	224 ± 9	49.1 ± 0.5
1:1	207 ± 10	42.9 ± 1.7	230 ± 26	46.1 ± 2.8
0.5:1	209 ± 16	36.0 ± 1.9	219 ± 13	32.1 ± 6.7

Values are mean averages ± SD of four different batches of particles.

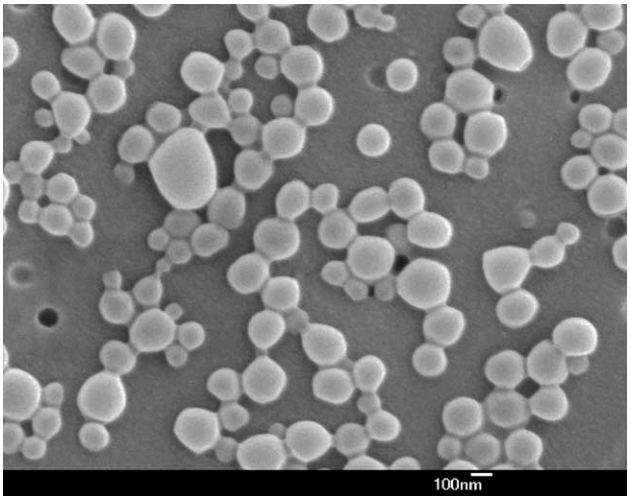

Fig. 1. Scanning electron micrograph of PLGA–PEI nanoparticles (PLGA–PEI ratio 10:1; PEI–DNA ratio 1:1) loaded with V1Jns–Ag85B plasmid DNA.

nanoparticles were taken up efficiently [14,32]. The stable nano-size of these particles makes them suitable for uptake by pulmonary epithelial cells and APCs, which is the first step for gene delivery. The zeta potential of PEI–DNA ratio of 5:1 was 52.3 and 53.5 mV for PLGA–PEI ratios 25:1 and 10:1, respectively, while PEI–DNA ratio of 0.5:1 resulted in lower zeta potential values of 36.0 and 32.1 mV (Table 1). These results indicate that the zeta potential was not affected by the PLGA–PEI ratio; however, it was influenced by the PEI–DNA ratio. The positive surface charge of the particles is a result of the orientation of PEI on the surface, since PLGA particles have a negative surface charge. The positively charged particle surface can facilitate adherence to negatively charged cellular membranes, inducing and increasing intracellular uptake.

SEM micrographs showed round particles, with a size distribution confirming the size measurements by PCS and particle morphology independent of PLGA–PEI ratio and PEI–DNA ratio (Fig. 1).

3.2. Loading efficiency of PLGA–PEI nanoparticles

Loading efficiency of the PLGA–PEI nanoparticles was above 99% for all PLGA–PEI ratios and PEI–DNA ratios tested (Table 2). Since our initial loading dose was 5 μg, the accuracy of this assay was high, considering 0.1% of the loading dose is still above the detection limit of the PicoGreen assay. This high and reproducible loading efficiency correlates with efficient surface adsorption that prevents material loss and suggests that even in the PEI–DNA ratio of 0.5:1, there is excess of nitrogen groups from the PEI over the phosphate groups of the DNA. Gautam et al. calculated that a 10:1 N/P ratio corresponds to 1.29:1 PEI–DNA weight ratio using the 25 kDa PEI [26], confirming that at 0.5:1 weight ratio there are still more nitrogen groups present than phosphate. Since we assume the PEI is physically attached to the nanoparticles surface, the exact number of nitrogen groups on the particle surface should be further evaluated in order to determine the exact N/P ratio.

Table 2
Loading efficiency of PLGA–PEI nanoparticles with different PLGA–PEI ratios and PEI–DNA ratios

PEI–DNA ratio	Loading efficiency (%) of PLGA–PEI ratio 25:1	Loading efficiency (%) of PLGA–PEI ratio 10:1
5:1	99.5 ± 0.1	99.5 ± 0.3
3:1	99.6 ± 0.2	99.4 ± 0.4
2:1	99.6 ± 0.2	99.7 ± 0.4
1:1	99.7 ± 0.1	100.0 ± 0.2
0.5:1	99.8 ± 0.1	100.0 ± 0.2

Values are mean averages ± SD three different batches of particles.

3.3. Intracellular localization of DNA adsorbed on PLGA–PEI nanoparticles and its protein expression

The intracellular distribution of the rhodamine-labeled DNA adsorbed to PLGA–PEI nanoparticles is shown in Fig. 2. The late endosomal distribution is visualized by immunostaining for LAMP-1 protein with an Alexa-conjugated secondary antibody (a). It was shown that after 6 h, the rhodamine-labeled DNA (b) is co-localized with the lysosomal signal (c). These results show that the particles were taken up by the lung epithelial cells and the DNA was located in the endo-lysosomal compartment. The culture conditions in the case of Calu-3 cells are of extreme importance since culture at air-interface (removal of apical culture medium one day after seeding) resulted in a morphology more representative of the in vivo situation in comparison to culture under submerged conditions (presence of culture medium on both sides of the cell monolayer) [33,34]. The fact that the nanoparticles were internalized and the DNA was transferred to the endo-lysosomal compartment despite the presence of cilia and mucus suggested that these particles were suitable to deliver DNA across mucosal surfaces of the lung. GFP protein expression was detected in Calu-3 cells, however, the signal was rather low. The fact that protein was detected suggested that a certain percentage of the particles could escape the endo-lysosomal compartment and DNA was able to enter the nucleus. Since the DNA is directly attached to the PEI on the particles surface and the PEI is more exposed to interaction with the endo-lysosomal membrane than the PLGA, we assume that the endo-lysosmal escape is governed by intrinsic characteristics of PEI. PEI is one of a few polycations that has a high transfection potential due to its intrinsic endosomolytic activity caused by high buffer capacity at virtually any pH. Upon acidification of the endo-lysosome, PEI causes osmotic swelling and subsequent endosomal rupture followed by the escape of PEI/DNA complexes into the cytosol [13,25]. Since PLGA–PEI nanoparticles delivered the DNA into the cells, escaped the endo-lysosmal compartment and resulted in protein

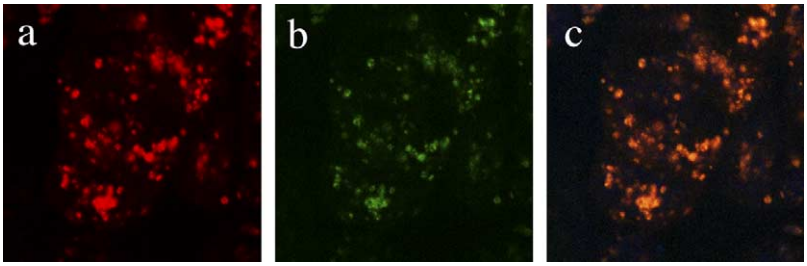

Fig. 2. Cellular internalization in calu-3 cells 6 h after application of PLGA–PEI nanoparticles loaded with rhodamine-labeled GFP encoding plasmid DNA. (a) immunofluorescence of anti-lysosomal-associated membrane protein-1 (LAMP-1) (red); (b) intracellular distribution of rhodamine-labeled DNA (green) and (c) superimposition of the confocal micrographs indicating co-localization of the DNA in the lysosomal compartments (orange–yellow).

expression, they should be regarded as a new promising DNA carrier for pulmonary delivery. This study evaluated qualitatively the intracellular pathway of the DNA loaded PLGA–PEI nanoparticles, however, quantitative analysis of protein expression in cell culture and using in-vivo models will be further investigated.

3.4. Cytotoxicity of PLGA–PEI nanoparticles in Calu-3 cells

In PEI–DNA ratio of 2:1, cell viability was measured to be 56.5% ± 4.8% and 61.5% ± 10.9% for PLGA–PEI ratios 25:1 and 10:1, respectively (Fig. 3). When the PEI–DNA ratio decreased, cell viability increased above 94% for the 1:1 ratio and full recovery was seen at the 0.5:1 ratio. This suggests that cell toxicity was independent on the PLGA–PEI ratio, but was markedly affected by the PEI–DNA ratio. Since an equal amount of DNA was given to all groups, the difference in cell viability reflected the amount of PLGA and PEI delivered. It was previously reported that PLGA particles did not significantly affect the viability of macrophages and DCs, even when loaded with a large number of microspheres and successive microsphere decay where acidic degradation products are formed [20]. However, it was shown in Calu-3 cells that higher PEI–DNA ratio correlates with higher cytotoxicity [35]. The results we present here show that cell toxicity can be reduced by using lower PEI–DNA ratio that will bear the same characteristics and loading efficiency as the higher PEI–DNA ratios. The non-toxic formulations will be further evaluated for their in-vitro and in-vivo ability to serve as DNA carriers for the purposes of gene therapy and vaccination.

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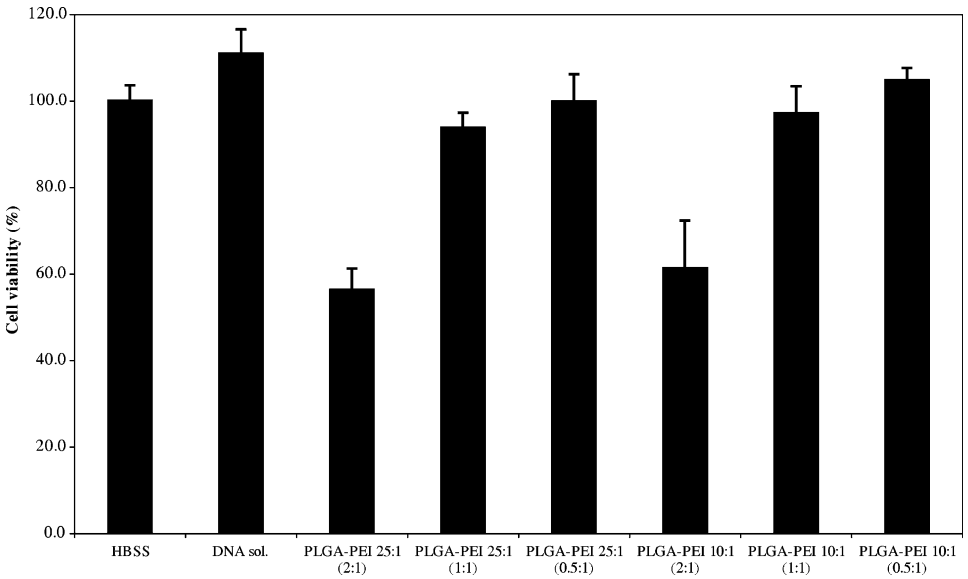

Fig. 3. Calu-3 cell viability after application of PLGA–PEI formulations. Values are the mean average ± SD of six wells applied with the same formulation. This is one representative experiment out of three performed.

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