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# Fast degrading polyesters as siRNA nano-carriers for pulmonary gene therapy

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## ARTICLE INFO

## ABSTRACT

A potential siRNA carrier for pulmonary gene delivery was assessed by encapsulating siRNA into biodegradable polyester nanoparticles consisting of tertiary-amine-modified polyvinyl alcohol (PVA) backbones grafted to poly(D,L-lactide-co-glycolide) (PLGA). The resulting siRNA nanoparticles were prepared using a solvent displacement method that offers the advantage of forming small nanoparticles without using shear forces. The nanoparticles were characterized with regard to particle size, zeta-potential, and degradation at pH 7.4 using dynamic and static light scattering. SiRNA release studies were performed and correlated to the nanoparticle degradation. In vitro knockdown of firefly luciferase reporter gene was used to assess the potential of the nanoparticles as siRNA carriers in a human lung epithelial cell line, H1299 luc.

The amine-modified-PVA–PLGA/siRNA nanoparticles form 150–200 nm particles with zeta-potentials of +15–+20 mV in phosphate buffered saline (PBS). Break down of the nanoparticles was seen within 4 h in PBS with sustained release of siRNA. These nanoparticles have achieved 80–90% knockdown of a luciferase reporter gene with only 5 pmol anti-luc siRNA, even after nebulization. Hence we conclude that amine-modified-PVA–PLGA/siRNA nanoparticles could be a promising siRNA carrier for pulmonary gene delivery due to their fast degradation and potent gene knockdown profile.

## 1. Introduction

Gene silencing by RNA interference (RNAi) offers new opportunities for the treatment of lung diseases such as lung cancer, cystic fibrosis, respiratory syncytial virus (RSV) and severe acute respiratory syndrome (SARS). Effective delivery of the siRNA to the site of action is hindered by many biological barriers i.e. bifurcations in the lung, mucociliary clearance, lung surfactant etc. [1,2]. Once across these barriers, a number of additional factors have to be taken into consideration. SiRNA is quickly inactivated by RNases and macrophages. Hence, siRNA has been condensed or encapsulated with non-viral siRNA carriers to afford its protection against host defences. The requirements for efficient lung deposition are generally defined as appropriate aerodynamic mean diameter of droplets or dry powders in the range of 1–5 μm [3], and sufficient stability of carriers to allow nebulization [4]. Once deposited in the lung, siRNA complex sizes should have a particle diameter less than 200 nm to avoid macrophage phagocytosis [5,6].

In an effort to enhance gene silencing, various non-viral carriers for siRNA delivery have been developed. Most of them are based on liposomal formulations or positively charged polycations, which are non-biodegradable [7,8]. Liposomal formulations have been shown to

be poor siRNA carriers for pulmonary gene delivery [7], possibly due to the surfactants inherent in the lung [9,10].

The design of biodegradable polymeric materials has become an important objective to avoid acute toxicity and accumulation of nano-carriers in the lung after administration [11]. SiRNA polyplexes formed with biodegradable poly(amino ester glycol urethane) were shown to display low cytotoxicity. Combined with a fast siRNA release, these siRNA polyplexes lead to efficient gene silencing in vitro [12]. Recent studies reported chitosan as a potential carrier for siRNA delivery, however none of them investigated the degradation in correlation with siRNA release and knockdown efficiency [13–15]. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles were most frequently used for encapsulation of peptides, proteins and pDNA. The breakdown of PLGA leads to acidic degradation products, lactic and glycolic acid, which can cause DNA degradation and damage [16]. These nanoparticles were not able to mediate pronounced DNA transfection efficiency possibly due to poor encapsulation, low cellular uptake, insufficient lysosomal escape, or as previously mentioned, DNA degradation [17].

In an attempt to overcome these drawbacks, branched biodegradable polyesters were designed by attaching hydrophilic, positively charged amine groups onto a hydrophilic water-soluble backbone consisting of poly(vinyl alcohol) which was subsequently grafted with multiple PLGA side chains [18]. In this study we focused on a derivative of poly(vinyl 3-(dialkylamino) alkylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly-(D,L-lactide-co-glycolide) containing diethylaminopropylamine (DEAPA) as amine function with

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composition DEAPA(68)–PVA–PLGA (1:10), abbreviated as P(68)-10. [19]. This polymer was found to be useful for pDNA containing nanoparticles [20] showing surprisingly high transfection efficiencies. The mechanism of transfection does not rely on the proton-sponge effect of the backbone [21] but rather on the rapid degradation rates. The biocompatibility of P(68)-10 was found to be superior to poly (ethylene imine) [22] and the acute toxicity and inflammatory response of P(68)-10 nanoparticles after pulmonary application was comparatively low [23]. The degradation rates and profiles are affected by the polymer structure and very rapid degradation rates can be achieved [24].

In this study we investigated the hypothesis that nanoparticles generated from rapidly biodegradable polymers could be advantageous for intra-cellular siRNA delivery. These nanoparticles were prepared by a modified solvent displacement method to encapsulate siRNA into biodegradable P(68)-10 under mild conditions. These nano-carriers were then characterized with respect to cytotoxicity, transfection efficiency and nebulization properties to assess their potential as pulmonary delivery system for siRNA.

## 2. Materials and methods

### 2.1. Materials

Biodegradable branched polyesters DEAPA-(68)–PVA–PLGA (1:10), abbreviated as P(68)-10, were synthesized and characterized as previously described [19]. The anti-luciferase siRNA: 5′-GAUUAUGUCCGGUUAUGUA-3′ (cat. No. D-002050-01-20) and siCONTROL Non-Targeting siRNA #3 (cat. no. D-001210-03-20) was purchased from Dharmacon (Lafayette, CO, USA). Lipofectamine 2000 was obtained from Invitrogen (CA, USA).

### 2.2. Preparation of nanoparticle suspension

Nanoparticles were prepared using a solvent displacement method, described by Jung et al. [25]. Briefly, 5 mg of P(68)-10 were dissolved in 500 µl of dry acetone with stirring for 48 h. In a sterile laminar flow hood, 20 µl siRNA (0.25 µg/µl) was dispersed into a 100 µl P(68)-10 acetone solution. For polymer ratios in the range of 6:1–20:1 N:P ratios, the amount of polymer ranges from 240–790 µg (P(68)-10 has 1 tertiary amine per 2513 Da). This mixture was immediately injected into a 400 µl of a 0.1% poloxamer (Pluronic F-68, Sigma-Aldrich, Munich, Germany) aqueous solution, whereupon the solution immediately turned opalescent. The acetone was removed from the nanoparticle suspensions by evaporation under laminar flow for 3 h or by a 50 mTorr vacuum pump for 20 min.

### 2.3. Efficiency of nanoparticle formation and siRNA encapsulation

SiRNA nanoparticles were filtered from the 0.1% poloxamer solution (1.0 ml) using preweighed 47 mm 0.02 µm Whatman filter discs. The filter discs were washed once with 2 ml of distilled water and allowed to dry under vacuum to constant weight to determine the concentration of the nanoparticles in suspension. The supernatant was analyzed for siRNA concentration by an ethidium bromide intercalation assay after 15 min incubation with 50 IU of heparin. Twenty µl of a 0.0125 mg/ml ethidium bromide solution was added. Fluorescence was measured using a fluorescence plate reader (LS 50 B, Perkin Elmer, Rodgau-Jügesheim, Germany) at excitation and emission wavelengths of 518 nm and 605 nm, respectively. Amount of siRNA was calculated by a calibration curve ranging from 0.0156 µg–0.75 µg siRNA. Experiments were carried out in triplicate.

### 2.4. Nebulization of nanoparticle suspension

The prepared nanoparticle suspension (200 µl) was pipetted directly onto the membrane of a sterilized Aeroneb Laboratory

Nebulizer. The aerosol was collected and condensed using a 1.5 ml Eppendorf tube pressed to the bottom of the nebulizer. Typical recoveries of the aerosol were >70%. Experiments were carried out in quadruplicate.

### 2.5. Laser diffraction of nebulized nanoparticle suspension

Nanoparticle suspension was diluted 1:5 with 0.9% NaCl solution. The diluted solution (600 µl) was placed into the Aeroneb Laboratory Nebulizer. A stream of nitrogen (10 l/min) was used to propel the aerosol through the laser beam of the Fraunhofer Laser Diffractor, and six measurements were taken of each formulation. Sodium chloride 0.9% served as control. Droplet sizes are expressed as mass median aerodynamic diameter (MMAD).

### 2.6. Measurement of particle size and zeta-potential

Hydrodynamic diameter of nanoparticle suspensions was measured by photon correlation spectroscopy (PCS) using the Zetasizer, Nano ZS, Malvern Instruments (Herrenberg, Germany). Nanoparticles were prepared in a total volume of 200 µl at different N/P ratios containing 200 pmol of siRNA. For determination of the zeta-potential the nanoparticle suspension was further diluted in either distilled water or HEPES buffered glucose (HBG) (5% Glucose buffered with 10 mM HEPES). All measurements are given as mean values of three independent runs and performed in triplicate.

### 2.7. Heparin competition assay

To study the nanoparticle stability against anions a heparin competition assay was performed. Heparin was dissolved in 10 µl pure water to obtain concentrations ranging from 0.05–2 IU per µg siRNA and added to 40 µl of nanoparticle suspension, Lipofectamine/siRNA or PEI 25 kDa/siRNA polyplex solution. After incubation for 15 min at RT all solutions were filled to a final volume of 180 µl and mixed with 20 µl of a 0.0125 mg/ml ethidium bromide solution. Fluorescence was measured using a fluorescence plate reader (LS 50 B, Perkin Elmer, Rodgau-Jügesheim, Germany) at excitation and emission wavelengths of 518 nm and 605 nm, respectively. Free siRNA was calculated by a calibration curve ranging from 0.0156 µg–0.75 µg siRNA. Results are given as means of triplicate measurements.

### 2.8. Nanoparticle degradation studies at pH 7.4

Nanoparticle suspensions (100 µl, 10:1 N:P ratio, containing Anti-Luc siRNA) were diluted 1:5 with phosphate buffered saline, pH 7.4 in a low volume Eppendorf UVette. Hydrodynamic diameter and light scattering (kilo counts per second, kcps) measurements were taken every 7 min at 37 °C using the Zetasizer, Nano ZS, Malvern Instruments. Each time point was measured in sextuplet, 10 s per measurement with total measurements up to 500 min.

### 2.9. SiRNA release studies at pH 7.4

For siRNA release studies at pH 7.4, 800 µl of nanoparticle suspensions (N:P ratio of 10:1) were diluted with phosphate buffered saline, pH 7.4 to a total volume of 4000 µl and incubated at 37 °C. At different time points (0 h, 1 h, 2 h, 3 h, 4 h, 6 h and 8 h) aliquots of 400 µl nanoparticle suspension were centrifuged at 16,000 ×g for 30 min at 4 °C. The pellet was separated from the supernatant and incubated with 50 IU. of heparin for 20 min. SiRNA was then quantified using the ethidium bromide intercalation assay as described in 'Efficiency of nanoparticle formation and siRNA encapsulation'. The values were calculated as mean±SD of three experiments.

### 2.10. In vitro luciferase gene knockdown experiments

H1299 *luc* cells, stably expressing luciferase, were cultured in RPMI medium, supplemented with 10% heat inactivated fetal bovine serum. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of $0.6 \times 10^4$ per well. Nanoparticles were prepared as described above and 5–20 µl (5–20 pmol siRNA) of the suspension were added to each well containing 100 µl fresh RPMI. After 4 h of incubation the medium was replaced and the cells were allowed to grow for 44 h. In addition to the anti-luc siRNA, a non-silencing siRNA sequence was used to ensure that the decrease in luciferase expression is due to the anti-luciferase siRNA and not to cytotoxicity effects or the vector. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used as a positive control for siRNA delivery and was prepared according to the manufacturer's protocol. Luciferase gene silencing activity was measured according to the protocol provided by Promega (Madison, WI, USA). Briefly, cells were lysed in 100 µl cell culture lysis buffer for 15 min. Luciferase activity was quantified by injection of 50 µl luciferase assay buffer, containing 10 mM luciferin, to 20 µl of the cell lysate. The relative light units (RLU) were measured with a plate luminometer (LumiSTAR Optima, BMG Labtech GMBH, Offenburg, Germany). All experiments were performed in quadruplicates and data were expressed as percentage of control (untreated cells).

### 2.11. Cytotoxicity determination by the MTT assay

Twenty-four hours before transfection, H1299 *luc* cells were seeded in 96-well plates at a density of $0.6 \times 10^4$ per well. Nanoparticle suspensions were serially diluted in RPMI/10% fetal bovine serum from 1 mg/ml to 0.004 mg/ml (total of nine concentrations). Quadruplicate measurements were made for each concentration using 100 µl of the diluted suspensions and allowed to incubate 24 h in the H1299 *luc* cells. Medium was removed and 200 µl RPMI plus 20 µl of 2 mg/ml MTT (3-(,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide) solution was added to each well and incubated for 4 h. MTT medium was removed and formazan was dissolved using 200 µl DMSO. Absorbance was measured at 560 nm and background at 670 nm was subtracted. Cell viability = nanoparticle concentration absorbance / blank cell absorbance × 100. Data are presented as a mean of four measurements. $LD_{50}$ was calculated using the Boltzman sigmoidal function from Microcal Origin® v 7.0 (OriginLab, Northampton, USA).

### 2.12. Membrane interaction studies by hemolysis assay

The hemolytic activity of nanoparticles and polyplexes was investigated as reported earlier [26]. Briefly, human erythrocytes were isolated from fresh citrated blood from a healthy volunteer by centrifugation at 850 ×g. Red blood cells were washed in PBS until the supernatant was clear. Erythrocytes were diluted with PBS to $5 \times 10^8$ cells/ml. Nanoparticle suspensions were prepared as explained in 'Preparation of nanoparticle suspension'. PBS and 1% Triton X-100 in PBS were used as controls for 0% lysis and 100% lysis, respectively. 50 µl aliquots of nanoparticle suspensions were mixed with 50 µl erythrocyte suspension. Samples were incubated for 30 min at 37 °C under constant shaking. After centrifugation at 850 ×g, supernatant was analyzed for hemoglobin release at 541 nm.

### 2.13. Statistics

Significance between the mean values was calculated using unpaired Student's *t*-tests. Probability values $P < 0.05$ were marked with *, $P < 0.01$ were marked with ** and $P < 0.001$ were marked with ***.

## 3. Results and discussion

### 3.1. Preparation of P(68)-10/siRNA nanoparticles

The P(68)-10 polymer was prepared and characterized as described by Wittmar et al. [19]. Physical properties of the P(68)-10 were $M_w = 1055$ kDa, $M_n = 374$ kDa, PDI = 2.82, and $T_g = 15$ °C.

Nanoparticle suspensions were prepared by dissolving the P(68)-10 polymer (Fig. 1, non-water soluble) into acetone and siRNA (dissolved in buffer) was then injected directly into the acetone solution. The solution immediately turned opalescent from nanoparticle formation, a phenomenon also known as the Marangoni effect [27]. The formation of nanoparticles by the solvent displacement method offered several benefits over traditional methods. For example, high speed mixing was avoided, which prevented shear forces on siRNA or the nanoparticles. Solvent displacement also allowed the scale of the nanoparticle formation to be modified from large batch sizes to small volumes such as 50 µl siRNA nanoparticles. The removal of acetone under reduced pressure allows faster preparation and did not affect the nanoparticles in terms of hydrodynamic diameter, zeta-potential, or transfection.

Investigation on efficiency of nanoparticle formation and siRNA encapsulation showed that for the P(68)-10 polymer at a 10:1 N:P (nitrogen to phosphate) ratio, 98 ± 2% formed nanoparticles that could be filtered by the 0.02 µm filter. The supernatant contained 7 ± 2% of the total siRNA which translates into an association of 93 ± 2% of the injected siRNA into the P(68)-10 nanoparticles. This compares favourably with other formulations such as chitosan coated PLGA nanoparticles where only 51% of siRNA was encapsulated [28]. The P(68)-10 nanoparticles had similar encapsulation efficiencies for oligonucleotides as liposomes (80–90%) [29] as well as chitosan–TPP encapsulated nanoparticles (100%) [15].

### 3.2. Physico-chemical properties P(68)-10/siRNA nanoparticles

Nanoparticle formulations were prepared by varying the ratio between the amine functions on the polymer backbone and the phosphate groups in siRNA, abbreviated as N:P ratio. The nanoparticle size and surface charges were characterized by dynamic light scattering (DLS) and zeta-potential respectively. With increasing N:P ratios, the sizes increased from 150 nm (N:P 6:1) to 225 nm (N:P 20:1), as seen in Fig. 2A. This observation is most likely due to the higher polymer concentration in the organic phase, when using higher N:P ratios. Quintanar-Guerrero and co-workers studied the factors controlling nanoparticle size by the solvent displacement methods [30,31]. Their results demonstrate that polymer concentration in the organic phase affects particle sizes—the higher the concentration, the larger the particle size. The siRNA concentration and acetone volume were constant, and only the concentration of the P(68)-10 polymer
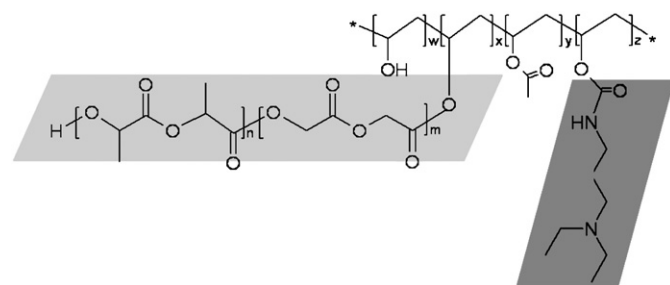


**Fig. 1.** DEAPA (68)–PVA–PLGA (1:10) polymer consisting of a 15K MW polyvinyl alcohol grafted to poly(D,L-lactide-co-glycolide) (light grey) with activation by 3-diethyl-amino-1-propylamine (dark grey) Physical properties for the specific polymer tested were $M_w = 1055$ kDa, $M_n = 374$ kDa, PDI = 2.82, and $T_g = 15$ °C (see Ref. [19] for details on synthesis and characterization).
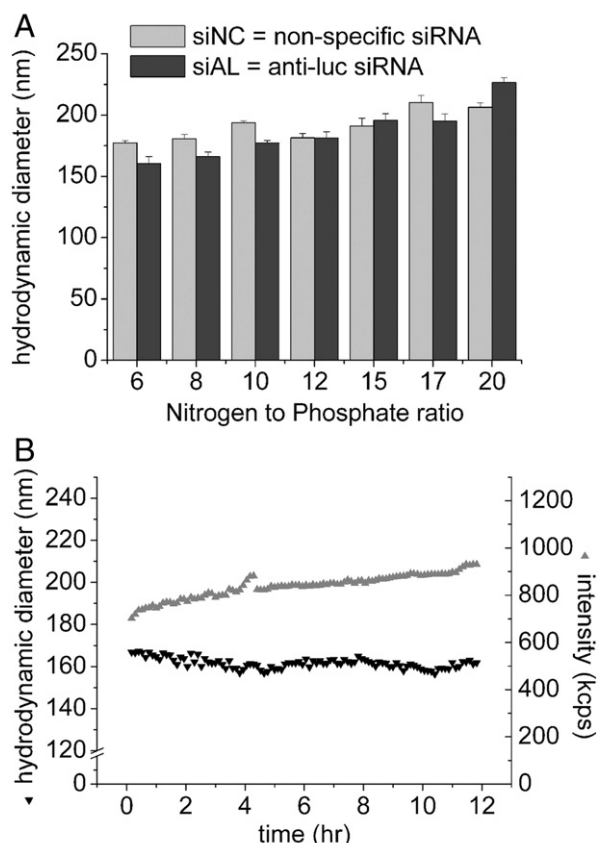
Fig. 2. Hydrodynamic diameters of P(68)-10/siRNA particles (A) as measured by dynamic light scattering and stability of 10:1 N:P nanoparticles (B) in 0. 1% poloxamer (Pluronic F68) solution.

### 3.3. Effects of nebulization on nanoparticle suspensions particle size and zeta-potential

Nebulization of the P(68)-10/siRNA nanoparticles was carried out using an Aeroneb Laboratory Nebulizer. This device uses an ultrasonic vibrating mesh to nebulize aqueous medium. Aerosol nebulizers have an advantage over air-jet nebulizers because of their higher output, which results in less shear forces due to nominal exposure time for oligonucleotides [4]. The ultrasonic energy has been known to alter or undermine aerosolized drug formulations [33,34]. Thus, P(68)-10/siRNA nanoparticles were characterized before and after nebulization, as seen in Fig. 3. The lowest ratio of N:P was affected the most by the nebulization. The 6:1 N:P ratio had a 37% increase in the hydrodynamic diameter and the zeta-potential had a larger increase in the standard deviation. The increases were most likely caused by ultrasound induced aggregation of the nanoparticles. For the 10:1 and 15:1 N:P ratios, no significant effects were seen on either particle size or surface potential (unpaired Student's $t$-test $P<0.05$).

### 3.4. Effects of aerosol droplet size by P(68)-10/siRNA nanoparticles

The size of the nebulized aerosol has importance for lung deposition of DEAPA P(68)-10/siRNA nanoparticles. The general size range for adequate deposition in the human lung ranges from 1–5 μm [3]. For comparison, saline and 0.1% poloxamer aerosols were measured by laser diffraction to determine the mass median aerodynamic diameter (MMAD) from the Aeroneb Laboratory Nebulizer, which was claimed to produce aerosols in the 2.5–4.0 μm range [35]. The median size ranges were 4.5±0.1 and 5.0±0.1 μm, respectively. The addition of the nanoparticle suspension (diluted with
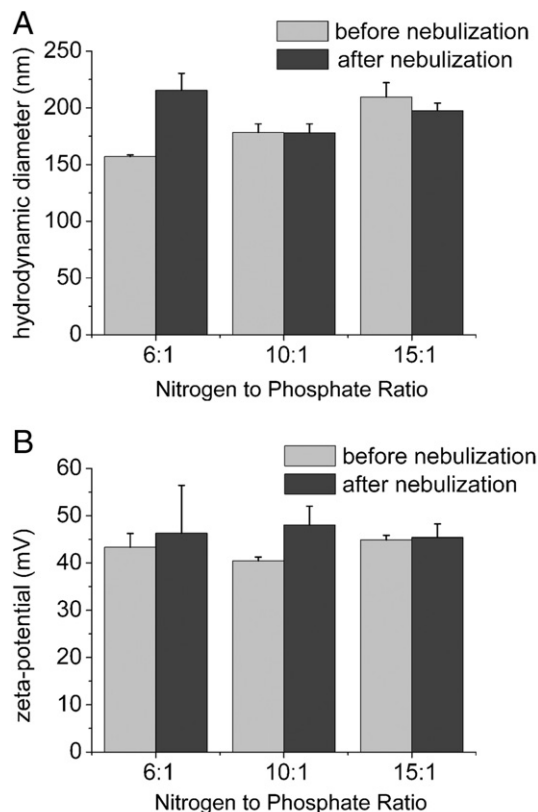
was changed to yield different N:P ratios. Overall, the type of siRNA (anti-luc siRNA or non-specific) did not influence the size, as expected.

Zeta-potentials for the P(68)-10 nanoparticles only varied by salt concentration. The zeta-potential did not vary with N:P ratio, presence or absence of siRNA, particle size or polymer injection methods (i.e. syringe versus plastic pipette tip injection). At solute/salt concentrations of <10 mM the zeta-potential was always seen in the range of 40–50 mV. When the nanoparticle suspension was diluted with isotonic saline solutions, the zeta-potential went down to 15–20 mV.

The stability of any siRNA carrier is always of utmost importance, as a possible therapeutic formulation would have to have the same integrity when formulated as when it would be used several hours later. To monitor the integrity of P(68)-10/siRNA nanoparticles, DLS measurements were recorded for 12 h on a freshly prepared sample of 10:1 N:P nanoparticle suspension (0.1% poloxamer, pH 5.2) containing the anti-luc siRNA. Fig. 2B demonstrated that nanoparticle suspensions did not aggregate or display gross degradation throughout the 12 h incubation period at 25°C. The hydrodynamic diameter remained constant, as the starting particle size of 166 nm was very close to the particle size of 161 nm at the end of the experiment. A slight increase in the scattering intensity was seen, but most likely resulted from solvent evaporation. The stability of these nanoparticles allowed a time flexibility for characterization and transfection experiments that is often not possible with polyplexes and lipoplexes. For example, the Lipofectamine 2000/siRNA lipoplexes need to be used within 30 min for optimum gene knockdown [32]. The P(68)-10/siRNA nanoparticles displayed no noticeable differences in transfection within the 12 h of preparation (data not shown). Future experiments will explore long long-term stability of the P(68)-10/siRNA nanoparticles and the effect on in vitro transfection.



Fig. 3. Effects of nebulization on the P(68)-10/siRNA (A) particle size and (B) zeta-potential.

saline) had little effect, as the MMAD was 4.4±0.3 μm. At this size range, a majority of the dosage would be available for therapeutic lung deposition.

### 3.5. Heparin competition assay

For efficient siRNA delivery into the cytosol the strength of electrostatic interaction between the siRNA and the transport vehicle is of major importance. Polymers with a high charge density such as PEI might form inseparable complexes with siRNA, impeding the release in the cytosol [36]. For high knockdown efficiency, siRNA formulations should be stable during cellular uptake but release siRNA in the cytosol, where it can bind to the RNA-induced silencing complex (RISC) and exert its biological activity [37]. To investigate the electrostatic interaction of the P(68)-10 and siRNA, a heparin competition assay was performed. PEI 25 kDa as a typical example for polyplexes and Lipofectamine for lipoplexes were included for comparison. In accordance with the low knockdown efficiency of PEI 25 kDa in H1299 luc cells, PEI displayed a strong electrostatic RNA binding. To release 30% of siRNA from PEI polyplexes, 0.5 IU heparin were required, and at least 1 IU heparin was necessary to release 70% of siRNA, as shown in Fig. 4. By contrast, P(68)-10/siRNA nanoparticles formed sufficiently stable nano-carriers for nebulization and endocytotic uptake (see *in vitro* biological activity) but the electrostatic binding was not as high, allowing sufficient release of siRNA in buffer and possibly in the cytoplasm. Only 0.05 IU heparin were necessary to release 30% of siRNA from the nanoparticles, and 0.5 IU heparin to release 70% siRNA. The much smaller binding affinity of P(68)-10 polymer was due to the type and density of the amine present. The tertiary dimethyl amine incorporated into P(68)-10 will be less protonated than the PEI primary amines [38] and therefore have less electrostatic affinity with the phosphate groups of siRNA. In comparison to PEI, the amine density is also about 50 times less, with 2500 Da/amine for P(68)-10 versus 43 Da/amine for PEI. These two properties combined ensure that the siRNA is weakly bound within the P(68)-10 nanoparticle, and upon P(68)-10 degradation, the siRNA is able to free itself from the degradation by-products. In the case of Lipofectamine, no siRNA could be released from the lipoplexes with heparin. However, recent studies showed that lipoplexes decomplex more easily than polyplexes, which can be explained by their fusogenic properties with cell membranes leading to the release of the siRNA [39].
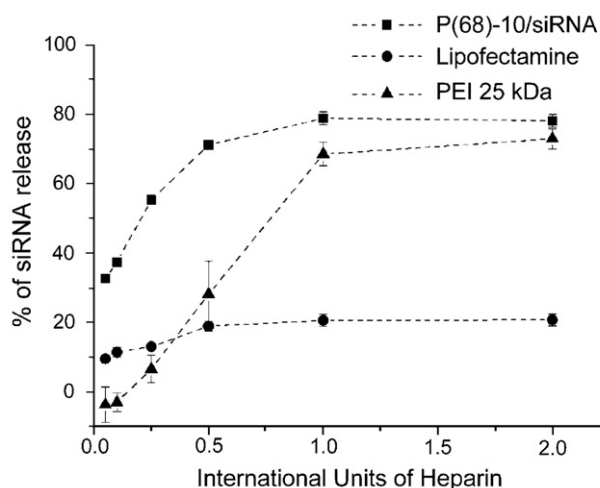


**Fig. 5.** Degradation (left axis) of P(68)-10/siRNA nanoparticles in pH 7.4 PBS correlated with siRNA release (right axis).

### 3.6. Nanoparticle degradation and siRNA release at pH 7.4

As mentioned above biodegradability is a key factor for long-term pulmonary application to avoid side-effects caused by accumulation [39–41]. The first breakthrough in pulmonary drug delivery was achieved with PLGA microparticles [42], due to their low toxicity and biodegradable character. The drawback of the PLGA microparticles was their slow degradation rate, which could vary from weeks to months. Wittmar et al. therefore synthesized a new class of DEAPA–PVA–PLGA polymers with a much faster degradation rate, by introducing hydrophilic amine groups [24]. To study the degradation profile of P(68)-10/siRNA nanoparticles under physiological conditions, static light scattering was used. The degradation of the P(68)-10 nanoparticles particles was shown to be dependent on the pH. Solutions of 0.1% poloxamer (pH 5.2, Fig. 2B) and 100 mM acetate buffer (pH 5.5, data not shown) both displayed $t_{1/2}$ of >1000 min. At physiological pH, degradation of the nanoparticles was complete in less than 300 min at pH 7.4 in phosphate buffered saline with a $t_{1/2}$ of 76 min as seen in Fig. 5. In accordance to the results obtained by static light scattering, siRNA release studies in phosphate buffer at pH 7.4 showed a 40% release during the first 2 h. After 4 h incubation a nearly complete release is achieved with 80% free siRNA. This rapid rate of siRNA release, correlated to the degradation of the siRNA carrier, has not been demonstrated for other siRNA carriers to our knowledge. A similar behaviour was shown by Tseng et al. with the formulations of biodegradable poly(amino ester glycol urethane)/siRNA polyplexes. They could show a maximum siRNA release after 14 h incubation in PBS pH 7.4 [12]. The rapid biodegradability does not only have a positive effect on biocompatibility but most likely contributes to a rapid release of the siRNA inside the cytosol after endocytic escape. Whereas in the acidic endosome (pH 5.0–5.5), there would be little to no degradation, and the siRNA would remain protected and encapsulated. Exactly how the P(68)-10/siRNA nanoparticles escape from the lysosome remains to be investigated in more detail. While lipoplexes are known to escape from endosomes by a fusion mechanism with the cell membrane [43], cationic polymers such as polyethylenimine (PEI) are thought to be released from endosomes by the so-called proton-sponge effect [44] and possibly local membrane damage [45]. Presently the escape mechanism of P(68)-10/siRNA nanoparticles is not known and remains to be determined in separate studies but we speculate that not only osmotic effects but also interactions with the endosomal membrane could play a role. This interaction would disrupt the membrane potential generated by membrane bound ATPases, and keep the pH near the extracellular and cytosol pH, where the degradation P(68)-10/siRNA is rapid and subsequent release of siRNA would find its way into the cytosol. Further studies are under way to clarify the mechanistic aspects of siRNA delivery using P(68) nanoparticles.



**Fig. 4.** Heparin displacement of siRNA from P(68)-10/siRNA nanoparticles, Lipofectamine 2000 siRNA lipoplexes, and 25,000 MW polyethylenimine (PEI)/siRNA polyplexes.
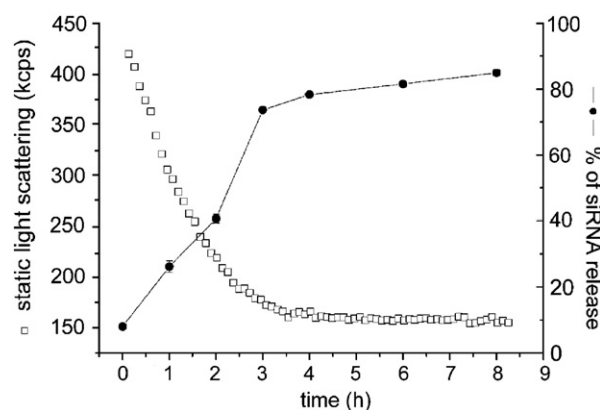
### 3.7. Cytotoxicity of P(68)-10/siRNA nanoparticles

To evaluate the toxicity of the pure P(68)-10 nanoparticles and P(68)-10/siRNA nanoparticles, the MTT assay and the RBC hemolysis assay were performed. PEI 25 kDa served as a comparison in both cases. As illustrated in Fig. 6A cells treated with pure P(68)-10 nanoparticles and P(68)-10/siRNA nanoparticles show relatively high cell viability. In comparison to PEI 25 kDa ($LD_{50}$=4.1 µg/ml), P(68)-10 nanoparticles display a 16-fold lower toxicity ($LD_{50}$=67.2 µg/ml). Encapsulating siRNA into the nanoparticles further decreases cytotoxicity, with a $LD_{50}$ of 179.2 µg/ml it shows a 3-fold difference from the unencapsulated nanoparticles. This effect is most likely due to the negative charges on the siRNA shielding or neutralizing some of the toxic P(68)-10 cationic charge.

As erythrocytes are one of the most used cellular membrane models, the RBC hemolysis experiment served as a model assay to investigate membrane interactions of P(68)-10/siRNA with cells [46]. As depicted in Fig. 6B 60% of the erythrocytes were hemolysed, when treated with 125 µg PEI 25 kDa. In contrast, incubation with 50 µl P(68)-10 nanoparticles without siRNA, equivalent to 125 µg DEAPA polymer, significantly reduced erythrocyte lysis and only led to 20% hemolysis. P(68)-10 nanoparticles with and without encapsulated siRNA were shown to be three times less hemolytic than PEI 25K, and the 6:1 P(68)-10/siRNA formulation displayed seven times less toxic membrane interaction.
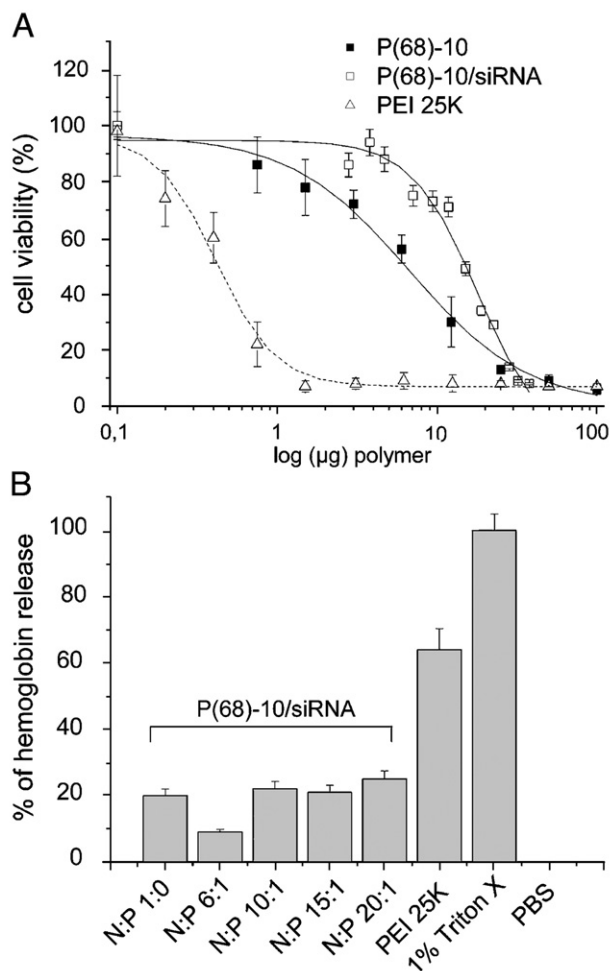


**Fig. 6.** Cytotoxic (A) and haemolytic (B) effects of P(68)-10 nanoparticles and P(68)-10/siRNA nanoparticles on H1299 cells, with 25,000 MW polyethylenimine (PEI 25 K) shown for comparison.

### 3.8. In vitro biological activity of P(68)-10/siRNA nanoparticles

The transfection efficiency of P(68)-10 nanoparticles was assessed in a human lung epithelial cell line, H1299 *luc*. This cell line was stably transfected to express firefly luciferase. Nanoparticle suspensions were formulated with anti-luc siRNA, which specifically targets firefly luciferase mRNA or a non-specific siRNA control that has at least four mismatches from any known human gene. Fig. 7 displays the results of seven different N:P ratios and three different siRNA doses. The more effective the siRNA carrier, the more luciferase mRNA cleavage and the less luciferase protein expressed. In contrast, using non-specific siRNA decrease in luciferase can only be observed if the P(68)-10/siRNA nanoparticles are affecting the cell metabolism or causing cytotoxicity. At the P(68)-10 nanoparticles/siRNA (5 pmol or 65 ng siRNA, Fig. 7A) formulation, the N:P ratios 10–20:1 displayed luciferase specific knockdown to levels of 15–20% compared to untreated H1299 *luc* cells, or control. Non-specific knockdown was low (>60% luciferase activity compared to control) and only seen in the largest N:P ratios. The 10 pmol nanoparticle suspensions achieved a higher level of anti-luc siRNA knockdown that was ~10% of control. The optimum N:P ratios (10 pmol or 130 ng siRNA, Fig. 7B) dosage were from 8–12:1 and were comparable to Lipofectamine 2000, a commercial transfection reagent. At the highest siRNA dosage tested, 20 pmol (Fig. 7C), anti-luc siRNA knockdown was the greatest with most of the values <5% of control. However, at 20 pmol siRNA, non-specific siRNA knockdown was seen in a dose dependent manner, which was most likely caused from P(68)-10/siRNA induced cytotoxicity.

Polyethylenimine (PEI) 25 kDa was used as a negative control. Known as a highly efficient standard transfection reagent for pDNA, it displayed less promising gene silencing in the case of siRNA. Strong electrostatic interactions with siRNA were shown using the Heparin Binding Assay and likely results in low knockdown efficiency due to incomplete siRNA release into the cytosol. The design of the P(68)-10/siRNA formulation allows for a more favourable release of siRNA, due to a number of factors. A highly positive surface charge of 15–20 mV ensures the binding of the nanoparticles to the negatively charged cell membrane, where uptake can take place. Once inside the endosomes, interaction of the positively charged hydrophobic nanoparticles with the endosomal membrane, in a similar way as it has been hypothesized for PEI [45], would lead to destabilization and local membrane damages. Water influx followed by neutralization of the acidic lysosomal environment would cause hydrolytic and enzymatic degradation of the PLGA chains into its by-products. As a result the osmotic pressure increases, thereby leading to further water influx and ultimate rupture of the endosomes [47]. In the cytosol (pH 7.4) degradation of the P(68)-10/siRNA nanoparticles is fast and subsequent release of the siRNA can occur. Rapid uptake and endolysosomal release in less than 10 min is also seen with PLGA nanoparticles [48]. These factors most likely contribute to the high luciferase knockdown seen with the small siRNA dosages of 5 and 10 pmol.

### 3.9. Effects of nebulization on in vitro biological activity of P(68)-10/siRNA nanoparticles

Nebulization of the P(68)-10/siRNA nanoparticles has been shown to effect the size and standard deviation of the nanoparticle suspension at low N:P ratios (see Fig. 3). This was also seen in the transfection of siRNA. Fig. 8 displays the results of two N:P ratios, 6:1 and 10:1, that were tested for effects after nebulization and condensation. For the non-specific siRNA, no effects are seen for either N:P ratio or siRNA dosage. From this data, it can be concluded that nebulization had no effect on the cytotoxicity properties of the nanoparticle suspensions. For the 6:1 N:P ratio, nebulization caused a significant decrease in both the 5 and 10 pmol siRNA dosages (unpaired Student's *t*-test). On average, there was a 20% increase in

luciferase knockdown activity after nebulization for the 6:1 N:P ratio. A cause for the decreased siRNA transfection observed after nebulization for the 6:1 N:P ratio might be linked by the results seen for the particle sizes. After nebulization the mean particle size increased from 157 nm to 215 nm. The increase in size of the nanoparticle suspension should not make much difference, as the 15:1 N:P ratio has a similar



Fig. 8. Knockdown of luciferase activity before and after nebulization of P(68)-10/siRNA nanoparticles. Statistics done with unpaired Student's *t*-test, probability values, $P<0.05$ were marked with *, and $P<0.01$ with **.
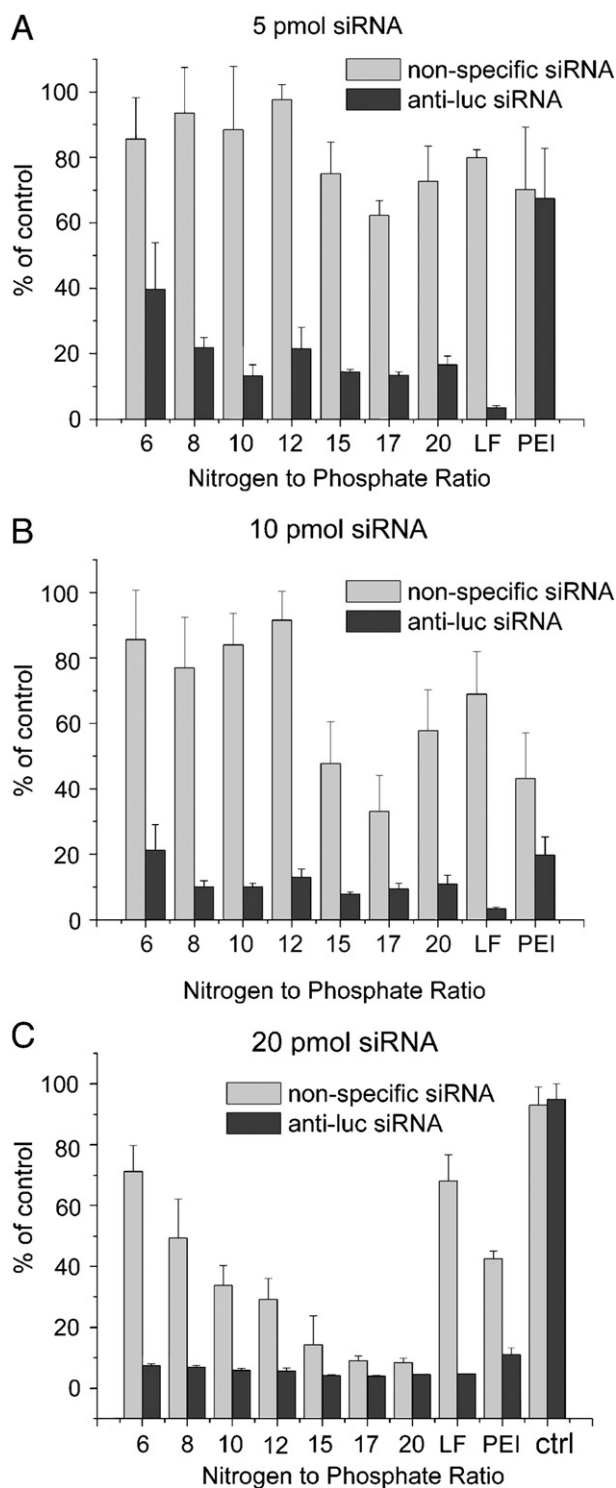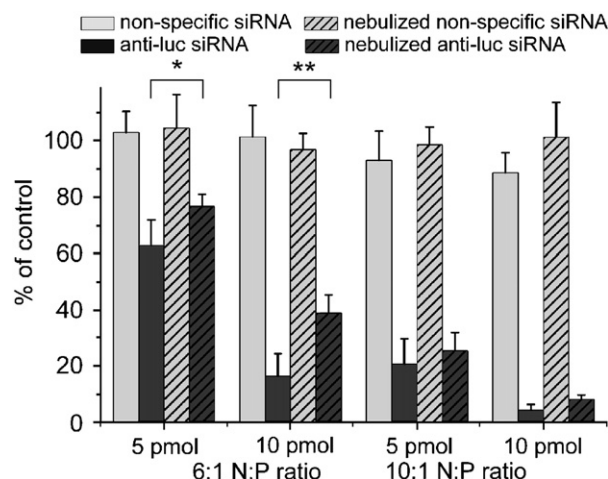


Fig. 7. The luciferase knockdown of P(68)-10/siRNA nanoparticles 5 (A), 10 (B), and 20 (C) pmol. LF = Lipofectamine 2000/siRNA 4:1 w/w ratio. PEI = 25,000 MW polyethylenimine/siRNA 10:1 N:P ratio.

particle size of 200–210 nm and transfection results were not affected after nebulization (data not shown). Instead, the vibrating energy of the nebulizer may have a more disruptive behaviour for the smaller 6:1 N:P, due to the smaller particle sizes, the increased proportion of siRNA it is carrying, or both. This disruptive effect possibly leads to less siRNA encapsulated or nanoparticles that degrades faster than observed, and therefore less siRNA delivered to the H1299 *luc* cells.

For the higher N:P ratio of 10:1, the nebulization had no effect on the siRNA knockdown of luciferase. Both formulations, before and after nebulization, had luciferase knockdown of >90%. This data, in consideration with aerosol droplet sizes of 4.5 μm, demonstrates the potential of P(68)-10/siRNA nanoparticles with ratios of 10:1 or higher to act as siRNA carriers for potential pulmonary delivery.

## 4. Conclusion

The design and formulation of siRNA carriers for gene therapy in the last few years have led to a broad panel of various liposomal and cationic polymer vectors. Liposome-mediated delivery showed efficient knockdown *in vitro* but were inefficient when applied *in vivo* via instillation [7]. Among the polymeric vectors, derivatives of PEI such as degradable oligo-ethylenimine, pegylated PEI and low molecular weight fraction of PEI have been used as siRNA carriers [8,49,50]. These siRNA carriers and others have slowly elucidated what is necessary for an ideal carrier. An ideal siRNA carrier for aerosol gene therapy needs to meet the following requirements: i) protection of the siRNA against high shear forces during nanoparticle formation and nebulization ii) fast degradation rate into non-toxic by-products at the target site iii) release of siRNA into the cytosol for effective gene silencing. The design of the branched polyester, consisting of an amine-modified PVA backbone grafted with PLGA chains, was tested to see if this polymer could meet the requirements needed for use as a siRNA carrier. SiRNA was shown to be encapsulated or absorbed with greater than 90% efficiency into the nanoparticles. The encapsulated siRNA was protected and remained intact during nanoparticle formation and after nebulization. Particle size and transfection results both demonstrate that the anti-luc siRNA remained active against luciferase as long as N:P ratios of at or above 10:1 were formulated. Breakdown of the nanoparticles within 4 h, shown by static light scattering, leads to a rapid release of the siRNA. Though this could be challenging for *in vivo* gene delivery as most physiological fluids are also within this rapid degradation pH range, the high surface potential of the P(68)-10/siRNA nanoparticles makes it likely that it will be taken into the lung cells quickly upon aerosol deposition. Nebulization

of the nanoparticle suspension had only minor effects on the *in vitro* biological activity at the lowest N:P ratio of 6:1. This issue could be easily overcome with an increase in the N:P ratio. At an N:P ratio of 10:1 the nebulized P(68)-10/siRNA nanoparticles displayed comparable knockdown efficiency to the non-nebulized samples. Only small siRNA dosages of 5 and 10 pmol were necessary to achieve a luciferase knockdown of 80–90% with minor to no cytotoxicity. The combined features of fast degradation combined with low cytotoxicity, nanoparticle stability during nebulization, and high specific knockdown make the P(68)-10 polymer a promising siRNA carrier for pulmonary gene therapy and future *in vivo* studies.

## Acknowledgements

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