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Degradable PEG-folate coated poly(DMAEA-co-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression

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ABSTRACT

A new cationic biodegradable polyphosphazene was developed, bearing both pendant primary and tertiary amine side groups, poly(2-dimethylaminoethylamine-co-diaminobutane)phosphazene (poly(DMAEA-co-BA)phosphazene). PEG and PEG-folate were coupled to polyplexes based on this poly(DMAEA-co-BA)phosphazene, leading to small (size 100 and 120 nm, respectively) and almost neutral particles. *In vitro* tissue culture experiments showed a low cytotoxicity of both uncoated and coated polyplexes. However, the PEG coated polyplexes showed a 2-fold lower transfection activity in OVCAR 3 cells as compared to the uncoated polyplexes. On the other hand, the PEG-folate coated polyplexes had a 3-fold higher transfection than the PEGylated polyplexes. When free folate was added to the transfection medium, only the transfection activity of the targeted polyplexes was reduced, indicating internalization of the targeted PEG polyplexes via the folate receptor. Confocal laser scanning microscopy confirmed a lower binding and uptake of the PEGylated polyplexes by OVCAR-3 cells when compared to uncoated and folate-PEGylated polyplexes. While uncoated polyplexes induced aggregation of erythrocytes at polymer concentrations of 0.09 μg/mL, the PEGylated systems could be incubated at ten times higher concentration before aggregation occurred indicating excellent shielding of the surface charge of the polyplexes by grafting of PEG. In conclusion, the targeted delivery of poly(DMAEA-co-BA)phosphazene bases polyplexes and their improved compatibility with erythrocytes makes them interesting for *in vivo* applications.

1. Introduction

A carrier system needs to fulfill the following requirements to be a promising candidate for *in vivo* gene delivery. The carrier should be able to efficiently accumulate in specific target tissues with minimal toxicity and immunogenicity, and deliver the intact gene into the nucleus of target cell to get high levels of gene expression. Viral vectors are the most efficient gene delivery systems known so far, however, they also have some severe drawbacks, like the induction of an

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immune response, in particular after repeated administration, possible recombination with wild-type viruses, limitations in the size of inserted DNA and oncogeniticity (McTaggart and Al-Rubeai, 2002; Gorecki, 2001; Schreier, 1994; Byrnes et al., 1995). Synthetic carriers such as polymers, therefore, have become an attractive alternative due to their relative safety and their lack of restraints on the size of the plasmid DNA to be delivered (Felgner and Rhodes, 1991; Curiel et al., 1991). Among the most studied polymers are pEI, pLL and pDMAEMA (Asayama et al., 1997; Boussif et al., 1995; van de Wetering et al., 1997). However, these polymers are either non-biodegradable (pEI and pDMAEMA) or show low transfection activity (pLL). In recent years there has been an increasing interest in biodegradable polymers, like polyesters, polyphosphazenes, degradable pEI, polyphosphoesters (Luten et al., 2003, 2006, 2007; Kim et al., 2005; Zhong et al., 2005; Christensen et al., 2006) as nonviral gene delivery vectors. Recently, the biodistribution and *in vivo* transfection efficiency of polyplexes composed of plasmid DNA and p(DMAEA)phosphazene were investigated after intravenous administration in tumour bearing mice (de Wolf et al., 2005). Polyplexes based on poly(DMAEA)phosphazene were shown to have a preferential tumor gene expression (organ gene expression <1/100 of tumour gene expression). This observed selectivity mediated by the p(DMAEA)phosphazene polyplexes could enable the application of this polymer to deliver therapeutic genes to tumours. However, the polyplexes were rapidly cleared from the circulation (<7% ID, at 60 min after administration). One way of improving the circulation kinetics of colloidal gene delivery particles is by PEGylation (Verbaan et al., 2004; de Wolf et al., 2007b). However, a major drawback of PEGylation of polyplexes is their lower transfection activity due to the loss of non-specific binding to the target cell. Introduction of targeting moieties on their surfaces is an interesting option to restore the transfection potential of poly(DMAEA)phosphazene-DNA polyplexes.

Targeting of the folate receptor, a glycopolypeptide with a high affinity ($K_d < 10^{-9}$ M) for folic acid and the physiologic circulating form of the vitamin, N5-methyltetrahydrofolate (Antony, 1996; Guo et al., 2006; Wang and Hsiu, 2005) had received much attention in recent years, since the folate receptor has been shown to be over expressed in human cancer cells and in addition, folic acid is a relatively small molecule (MW 441 Da) which does consequently have only limited effects on the dimensions of the carrier system (Corona et al., 1998; Elnakat and Ratnam, 2004; Sudimack and Lee, 2000). To improve the availability of folate for receptor-binding, attachment to a poly(ethylene glycol) (PEG) spacer was found effective for the targeted delivery liposomes (Lee and Low, 1994; Reddy and Low, 2000; Lee and Kim, 2005) and similar findings were reported for polyplexes (Seymour et al., 1991). As aforementioned, PEGylation of polyplexes has been shown to significantly improve their pharmacokinetics after intravenous administration (Verbaan et al., 2004; Cho et al., 1998; Ogris et al., 1999). On the other hand, PEGylated cationic polymers have been shown to exhibit less satisfactory DNA-condensing properties (Erbacher et al., 1999; Zuidam et al., 2000) which can be overcome by conjugating PEG to preformed polyplexes (post-PEGylation) (Verbaan et al., 2004; van Steenis et al., 2003; Blessing et al., 2001). To use post-PEGylation in the case of polyphosphazene-based polyplexes requires a polymer with a functional group that is able to covalently bind the PEG chain with or without a targeting ligand. In this paper, we report the synthesis, characterization of a polyphosphazene capable of post-PEGylation and the *in vitro* evaluation of the corresponding PEGylated polyplexes with and without folate targeting groups in OVCAR-3 cells.

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene $(NPCl_2)_3$ (>99.99%), calcium sulphate dihydrate, folic acid, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimid hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), benzyl chloroformate (Z-Cl), di-*tert*-butyl dicarbonate (BOC_2O), 1,4-diaminobutane (BA) and 2-dimethylaminoethylamine (DMAEA) were purchased from Aldrich (Zwijndrecht, The Netherlands) and were used as received. COOH-PEG_{5000}-NH_2·HCl and PEG_{5000}-NHS were purchased from Nektar (Huntsville, AL, USA). 1,4-Dioxane (Biosolve, Valkenswaard, The Netherlands) was dried over and distilled from sodium metal using benzophenone as an indicator. INF-7, a 24 amino acid containing peptide with fusogenic activity derived from the influenza virus, was synthesized via standard Fmoc solid-phase synthesis (Plank et al., 1994). PEI (branched, 25 kDa) was purchased from Sigma (Zwijndrecht, The Netherlands). PDMAEMA (M_n = 92 kDa) was synthesized by a radical polymerization of 2-dimethylaminoethyl methacrylate in an aqueous solution (Cherng et al., 1996). The plasmid pCMVLacZ, containing a bacterial LacZ gene preceded by a nuclear localization signal under control of a CMV promoter, was purchased from Sanvertech (Heerhugowaard, The Netherlands). The plasmid DNA was labeled with Cy5 dye using the Mirus *Label It* nucleic acid labeling kit (Sopachem, Wageningen, The Netherlands) and the cells were embedded in FluorSave Reagent (Calbiochem, San Diego, USA) for confocal laser scanning microscopy (CLSM) experiments.

2.2. Synthesis of poly(DMAEMA-co-BA)phosphazene

Mono-Z-diaminobutane was prepared as shown in Fig. 1, according to literature procedures (Atwell and Denny, 1984). In detail, a solution of di-*tert*-butyl dicarbonate (100 mmol, 21.8 g) in dioxane (400 mL) was added over a period of 5 h to a solution of 1,4-diaminobutane (700 mmol, 70.4 mL) in dioxane (400 mL) The mixture was stirred overnight at room temperature and the solvent was removed *in vacuo*. Water (400 mL) was added to the residue and stirred for 20 min. The insoluble bis-substituted product was removed by filtration. Next, the filtrate was extracted with DCM (3× 200 mL) and the organic layers were washed with brine (200 mL), dried with $MgSO_4$, and concentrated *in vacuo* to afford 17 g N-BOC-1,4-diaminobutane (88%). ^{1}H NMR ($CDCl_3$, δ in ppm) 4.94 (BOC-N*H*, 1H, br), 3.03 (CH_2, 2H, br), 2.64 (CH_2, 2H, br), 1.98 (NH_2, 2H, br), 1.42 (CH_2CH_2, 4H, br), 1.38 (CH_3, 9H, s).

Next, N-BOC-1,4-diaminobutane (13.9 mmol, 3.5 g) was reacted with Z-Cl (1.1 equiv., 3.2 g) under Schotten–Baumann

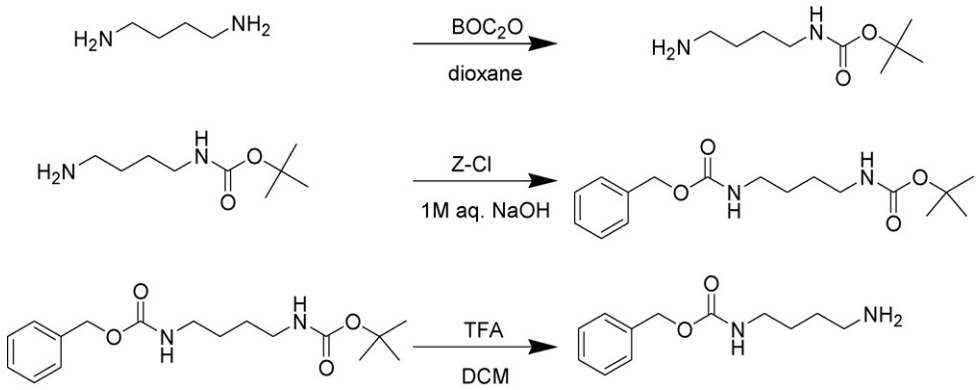

Fig. 1 – Synthesis of mono-Z-diaminobutane.

conditions (1 M aq. NaOH, 1.1 equiv., 0–20 °C, 16 h) to give the required unsymmetrical dicarbamate (6.0 g, 82%). ^{1}H NMR ($CDCl_3$, δ in ppm) 7.35 (C*H*, 5H, s), 5.09 (CH_2, 2H, s), 4.69 (N*H*, 2H, br), 3.21 (CH_2, 2H, br), 3.12 (CH_2, 2H, br), 1.51 (CH_2CH_2, 4H, br), 1.43 (CH_3, 9H, s).

The dicarbamate was subsequently selectively deprotected with TFA in DCM (1:1) (0 °C, 1 h) to yield mono-Z-diaminobutane in 76%. ^{1}H NMR ($CDCl_3$, δ in ppm) 7.34 (C*H*, 5H, s), 5.08 (CH_2, 2H, s), 4.95 (N*H*, 1H, br), 3.18 (CH_2, 2H, br), 2.73 (CH_2, 2H, br), 1.98 (NH_2, 2H, br), 1.51 (CH_2CH_2, 4H, br).

A polyphosphazene co-polymer with pendant primary and tertiary amines was synthesized as shown in Fig. 2. Poly(dichloro)phosphazene was synthesized via bulk polymerization. A glass ampoule was treated with TMS-Cl, cleaned and dried in a vacuum oven prior to use. Next, the ampoule was filled with hexachlorocyclotriphosphazene (4.0 g, 70 mmol P-Cl), and $CaSO_4 \cdot 2H_2O$ (10 mg, 0.06 mmol) and the ampoule was then sealed under high vacuum. The reaction mixture was heated to 250 °C for 6 h. The polymer/trimer mixture was then cooled to room temperature and diluted with 1,4-dioxane (200 mL), containing mono-Z-diaminobutane (1.6 g, 7.0 mmol, NH_2/P-Cl ratio 0.1) and triethylamine (20 mL, in excess). The reaction mixture was stirred for 2 days at room temperature followed by the addition of 2-dimethylaminoethylamine (DMAEA) (19 mL, 0.17 mol). The mixture was stirred for 6 days followed by removal of the formed triethylamine-HCl salt by filtration and the filtrate containing the polymer was concentrated under reduced pressure. Next, the polymer was dissolved in 100 mL MeOH and the Z-group was removed using Pd/C (1.0 g, 10%) and H_2 gas overnight at room temperature. Subsequently, the reaction mixture was concentrated and the polymer was dialyzed (cellulose acetate with a molecular weight cut off of 12,000–14,000 Da) against water for 3 days. The polyphosphazene was collected by lyophilization (yield: 300 mg, 4%).

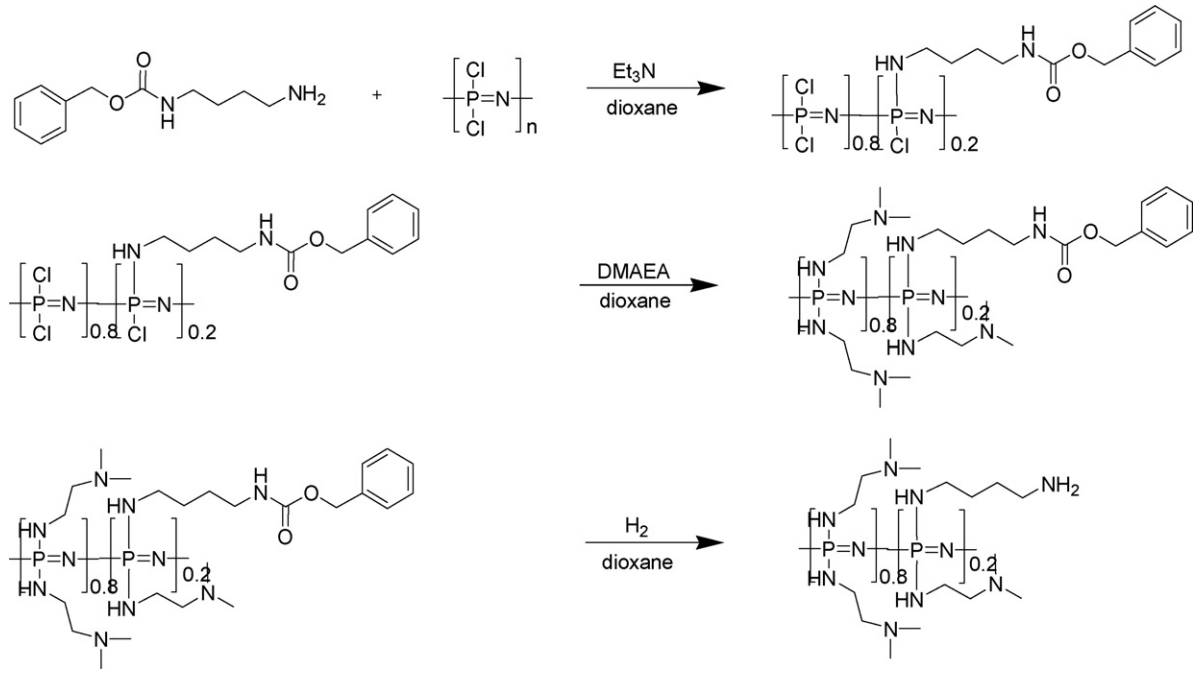

Fig. 2 – Synthesis of poly(DMAEA-*co*-BA)phosphazene.

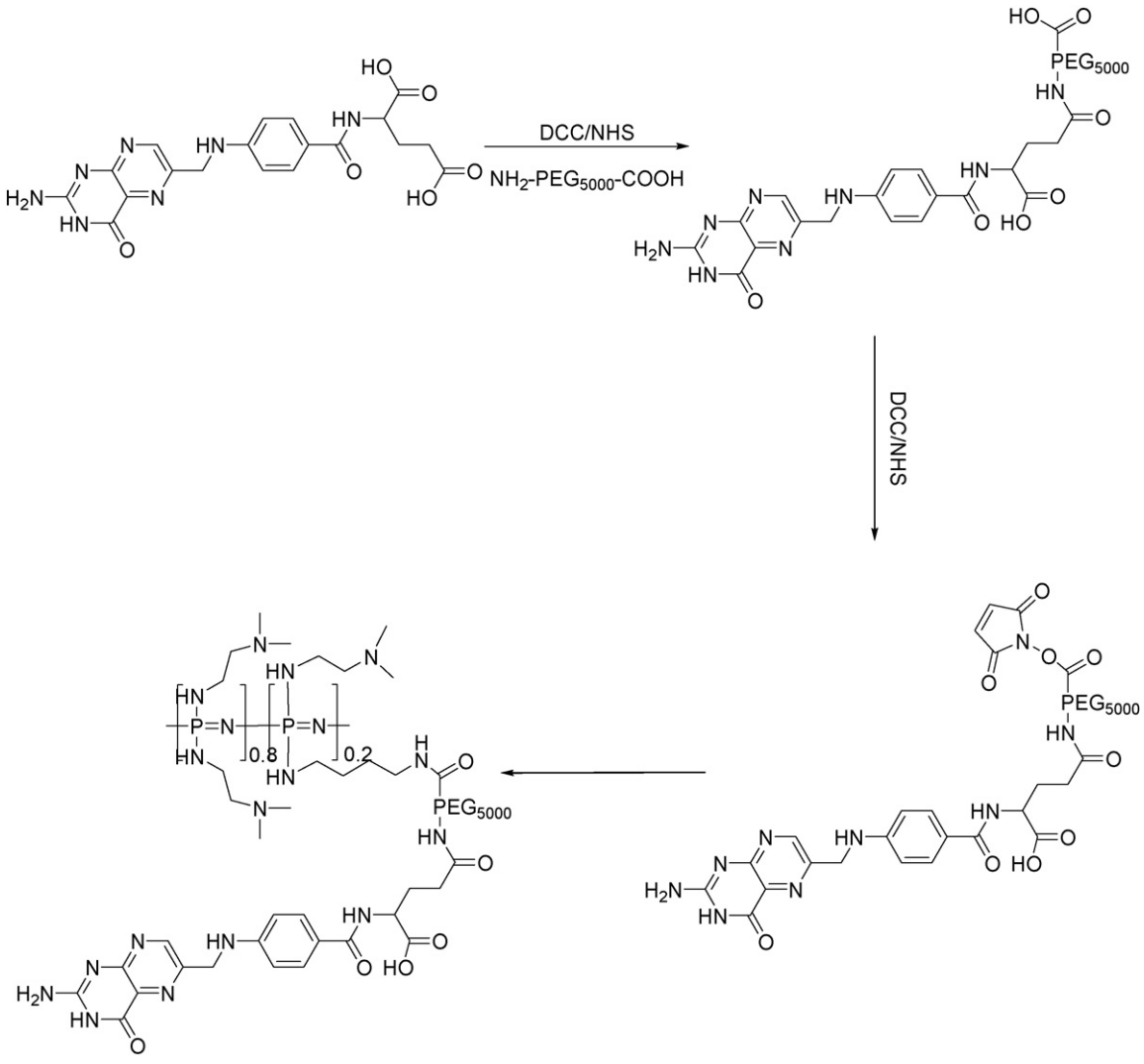

Fig. 3 – Synthesis of PEG-folate-NHS and coupling to poly(DMAEA-co-BA)phosphazene.

2.3. *Synthesis of NHS-activated folate-conjugated PEG*

Folate *N*-hydroxysuccinimidyl ester (folate-NHS) was prepared according to a literature procedure (Fig. 3) (Cho et al., 2005). In brief, folic acid (65 mg) was dissolved in DMSO (2.6 mL) to which triethylamine (0.05 mL) was added. After addition of *N*-hydroxysuccinimide (NHS) (38 mg, 2.2 equiv.), and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimid hydrochloride (EDC) (30 mg, 1.1 equiv.), the mixture was stirred in the dark for 18 h. Folate-NHS was coupled to NH_2-PEG-COOH as follows: the folate-NHS solution was added to PEG (350 mg, 70.0 μmol) dissolved in a mixture of DMSO (2.5 mL and triethylamine (0.1 mL), after which the mixture was stirred overnight. The folate-PEG-COOH was dialyzed extensively against deionized H_2O. Insoluble products including unconjugated folic acid were removed by filtration through a 0.2-μm filter (Millipore, Bedford, MA). The soluble product was collected and freeze-dried. Next, folate-PEG-COOH was reacted with EDC/NHS (10 equiv. to COOH) in DMSO to yield folate-PEG-NHS. The conjugate was purified by precipitation in diethylether, collected and stored at −20 °C.

2.4. *Characterization of poly(DMAEMA-co-BA)phosphazene*

^{1}H NMR spectra were recorded on a Varian G-300 300 MHz spectrometer (Varian, Palo Alto CA, USA) and ^{31}P NMR spectra on a Varian Inova 500 MHz spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are given relative to tetramethylsilane or phosphoric acid (85%) as an external reference; measurements were performed in CD_3OD.

Weight average molecular weight (M_w) and number average molecular weight (M_n) of the synthesized polymers were determined by gel permeation chromatography (GPC), using a Viscotek VE 2001 system (de Wolf et al., 2007a). Two Shodex SB-804 M columns with a pre-column (Shodex OH-pak SB-G) were connected to a Triple Detector Array 302, equipped with a low and a right angle light scattering detector, a viscometer detector and a refractive index detector. The eluent was 0.3 M NaAc (pH 4.4) with a flow-rate of 1.0 mL/min (Jiang et al., 2006). The refractive index increment (dn/dc) of p(DMAEA)phosphazene was determined by injecting p(DMAEA) phosphazene solutions with different concentrations (1–10 mg/mL) directly into

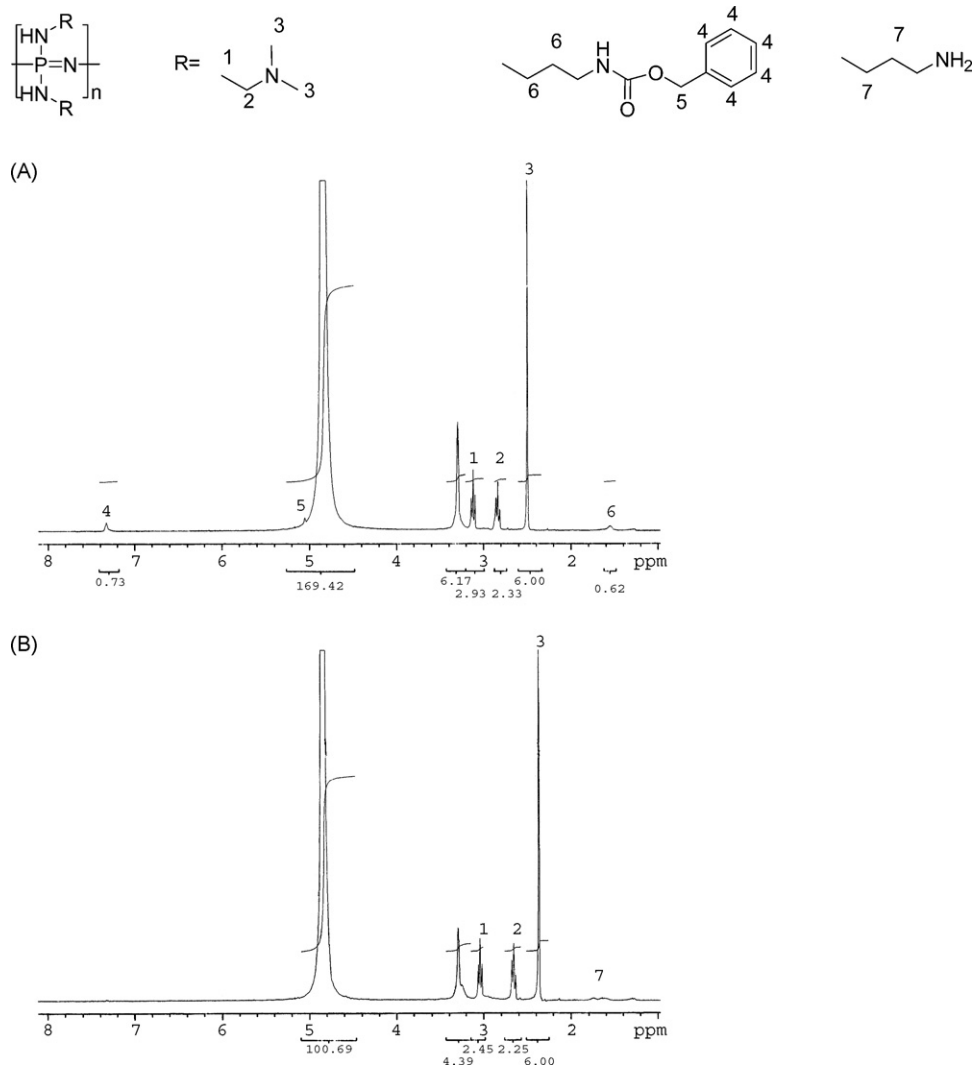

Fig. 4 – 1H NMR spectra of Z-protected p(DMAEA-*co*-BA)phosphazene (A) and p(DMAEA-*co*-BA)phosphazene (B).

the refractive index detector and was determined to be 0.159 mL/g. Molecular weight data analysis was performed by OmniSEC 4.1 software.

For the detection of the PEG conjugates a mesopor column was used; detection was done with a UV detector (363 nm). DMF/LiCl (10 mM) was used as the eluent with a flow rate of 0.7 mL/min.

The amount of primary amines in the polymer was determined spectrophotometrically by the ninhydrin assay (Romberg et al., 2006). Samples were prepared in 1 M sodium acetate buffer, pH 5.5, containing approximately 0.2 μmol NH_2/mL. Next, 1 mL of this polymer solution was taken and freshly prepared ninhydrin solution (2.0 g ninhydrin and 0.3 g hydrindantin dissolved in 75 mL of 2-methoxyethanol and 25 mL of 4 M sodium acetate buffer, pH 5.5) was added. The mixtures were vortexed and incubated for 15 min at 100 °C. After cooling to room temperature, the samples were diluted with 5 mL of 50% ethanol in water and the absorbance was measured at 570 nm with a PerkinElmer Lambda 2 UV/vis spectrophotometer. Glycine in a 1 M acetate buffer was used for calibration. The calibration curve was linear up to 0.5 μmol glycine/mL. As a control p(DMAEA)phosphazene, lacking primary amines, was used.

2.5. *PEGylation of polyplexes*

PEGylation of polyplexes was performed by adding DMSO solutions of either NHS-PEG-folate (53.9 mg/mL stock solution) or NHS-PEG (49.1 mg/mL stock solution) to the polyplexes, and incubating these for 15 min at room temperature. The polyplexes were prepared by adding 200 μL of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (5.0 mM, pH 7.4), containing 15 μg of plasmid DNA to 800 μL of HEPES buffer (5.0 mM, pH 7.4), with 90 μg of polymer (6:1, w/w, N/P ratio 18). The resulting dispersion was vortexed for 5 s and after 30 min incubation time at room temperature the size of the polyplexes was determined using DLS at 25 °C. To this dispersion 50 μL of either NHS-PEG-folate or NHS-PEG solution was added. The concentration of the PEG solutions was varied by diluting the stock solutions mentioned above to obtain different PEG/primary amine ratios.

2.6. Size and charge measurements of the different polyplexes

Z-average diameter and polydispersity of the polymer/plasmid DNA complexes were measured by dynamic light scattering (DLS) using a Malvern ALV CGS-3 system equipped with an argon-ion laser (488 nm, 10.4 mW) (uniphase). The ALV correlator software in combination with DTS (Nano) and ALVFilereader software was used. The instrument was calibrated with 100 nm poly(styrene) particles dispersed in water. In a typical experiment, 200 μL of HEPES buffer (5.0 mM, pH 7.4), containing 15 μg of plasmid DNA was added to 800 μL of HEPES buffer (5.0 mM, pH 7.4), with various amounts of polymer. The resulting solution was vortexed for 5 s and after 30 min incubation time at room temperature the size of the polyplexes was determined using DLS at 25 °C.

The ζ-potential of the polyplexes was measured at 25 °C using a Zetasizer Nano-Z (Malvern). The instrument was calibrated using a poly(styrene) dispersion with a known ζ-potential. In a typical experiment, 200 μL of HEPES buffer (5.0 mM, pH 7.4), containing 15 μg of plasmid DNA was added to 800 μL of HEPES buffer (5.0 mM, pH 7.4) with various amounts of polymer. The resulting solution was vortexed for 5 s and after 30 min incubation time at room temperature the surface charge properties of the polyplexes was determined.

2.7. Transfection studies

Transfection studies were done in OVCAR-3 cells as described previously using pCMV*lacZ* as reporter gene (van Steenis et al., 2003; Cherng et al., 1996). In brief, 96-well plates were seeded with cells at a density of 3×10^4 cm^{-2} 24 h before transfection. Polyplexes were prepared as follows: 150 μL of polymer solution in HBS was added to 50 μL of plasmid solution (50 μg/mL) in HBS, and after incubation for 30 min at room temperature, 5 μL of either PEG-NHS (9 mg/mL) or PEG-folate solution (10 mg/mL) in DMSO was added, and the polyplexes were incubated for another 15 min. Finally, 45 μL of INF-7 peptide (170 μg/mL) was added to the dispersion before transfer to the cells. After rinsing the cells with HBS, 100 μL of polyplex dispersion and 100 μL of culture medium were incubated with the cells for 1 h. After removal of the polyplex medium, fresh culture medium was added, and the cells were incubated for another 48 h. All transfection experiments were performed in two identical series in separate 96-well plates. One series was tested for reporter gene expression (β-galactosidase) by ONPG colorimetric assay; the other series was used to determine the number of viable cells using an XTT colorimetric assay. Transfection efficiencies were normalized to that of pDMAEMA polyplexes (polymer/DNA ratio of 3/1) in the presence of serum. Also, the transfection activity of polyplexes of PEI (branched, 25 kDa) in the presence of 5% serum was measured.

2.8. Confocal laser scanning microscopy studies

The uptake and intracellular presence of polyplexes with Cy5-labeled plasmid DNA was visualized via confocal laser scanning microscopy. This experiment with labeled DNA was essentially performed as described above for the transfections studies with the following modifications. A 16-well glass plate was used instead of a 96-well plate and after one hour incubation time, the cells were rinsed with phosphate buffered saline (PBS), fixed with 2.0% paraformaldehyde solution in PBS for 1 h at 4 °C and rinsed again with PBS. Cells were embedded in FluorSave Reagent and covered with a cover glass. Fluorescent and transmitted light microscope images of cells were taken simultaneously using a Leica TCS-SP microscope and analyzed using Leica TCS-SP Power Scan software (Leica Microsystems, Rijswijk, The Netherlands).

2.9. Polyplex-induced erythrocyte aggregation

Blood was collected from the vena cava inferior of female BALB/c mice. Heparin (approximately 50 Units per sample) was added to prevent blood coagulation. The blood samples were centrifuged for 12 min at 4 °C at 1200 × *g* and the pelleted erythrocytes were washed three times with HBS (5.0 mM Hepes, 150 mM NaCl, pH 7.4). Polyplexes were prepared as described before at a DNA concentration of 15 μg/mL. In a V-shaped 96-well plate 100 μL of polyplex dispersion were pipetted by serial dilution of the polyplex solution, leading to DNA concentrations ranging from 3.7 ng/mL to 15 μg/mL.

Subsequently, 50 μL of a 450 mM NaCl solution was added, followed by the addition of 50 μL of a 1% erythrocyte suspension in HBS. The erythrocytes were allowed to sediment overnight. Erythrocyte aggregation was scored visually.

3. Results and discussion

3.1. Synthesis of poly(DMAEMA-co-BA)phosphazene

A poly(DMAEMA-*co*-BA)phosphazene with pendant primary and tertiary amines was synthesized as shown in Fig. 2. The synthesis of poly(DMAEMA-*co*-BA)phosphazene was explored via several routes. The use of mono-BOC-diaminobutane and mono-BOC amino ethanol instead of mono-Z-diaminobutane was unsuccessful, because deprotection of the primary amine in CH_2Cl_2/trifluoro acetic acid 1:1 led to extensive chain scission. Therefore, the Z-group was investigated as protecting group to synthesize the aimed polymer. Direct mono-Z protection of 1,4-diaminobutane was practically impossible (yields were typically below 3% using Z-Cl in aq. NaOH/THF at 0 °C) while the di-Z protected diamine was formed predominately (Atwell and Denny, 1984). Mono-Z-diaminobutane was therefore synthesized following a known method that consisted of a route via mono-BOC-mono-Z-diaminobutane (Biagbrough et al., 1996). The mono-Z-protected diaminobutane was subsequently used to synthesize the Z-protected poly(DMAEMA-*co*-BA)phosphazene. First, mono-Z-protected diaminobutane was reacted with poly(dichloro)phosphazene in a 1:10 NH_2/P-Cl ratio. Then, an excess of DMAEA was used to substitute the remaining chloride atoms. Other strategies, like adding both substituents simultaneously or reversing the order of addition of the substituents were unsuccessful. ^{1}H NMR analysis showed (Fig. 4) that 12% of the side groups was Z-protected BA. This was also confirmed with a ninhydrin assay after the Z-group was successfully removed by Pd/C and H_2-gas (Fig. 4) (Romberg et al., 2006). The ^{31}P NMR spectrum of

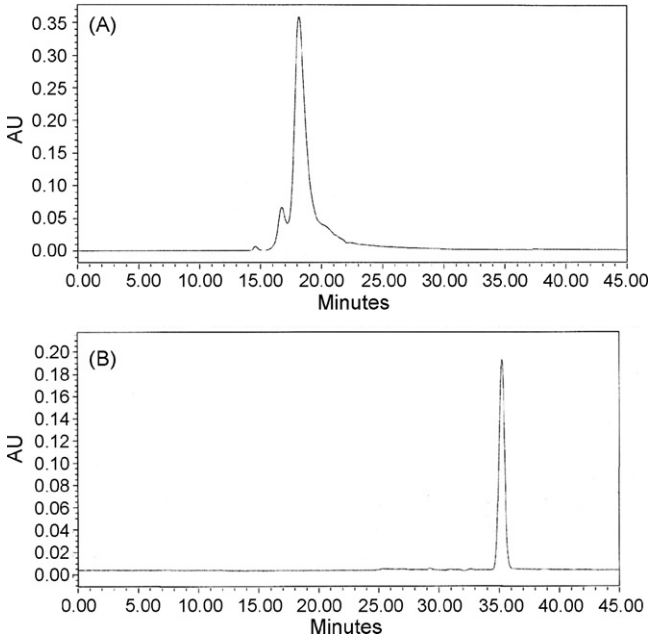

Fig. 5 – GPC results of the PEG-folate construct (A) and folic acid (B) at 363 nm. A physical mixture of folic acid and PEG did not cause a shift of the folate signal (data not shown).

the deprotected polymer showed only one signal at 1.7 ppm, indicating complete substitution of the chloride atoms and overlap of P-NH-BA and P-NH-DMAEA signal.

GPC analysis showed that the weight average molecular weight and the number average molecular weight of poly(DMAEA-*co*-BA)phosphazene were 20 and 8.5 kDa, respectively.

3.2. *Synthesis of NHS-activated folate-conjugated PEG*

To PEGylate preformed polyphosphazene-based polyplexes (so called post-PEGylation), an NHS-activated PEG folate conjugate was prepared according to a described method (Cho et al., 2005). GPC analysis was used to analyze the synthesized conjugate. GPC analysis showed that PEG-folate had a much shorter retention time than the free folic acid (Fig. 5A and B), whereas a physical mixture of folate and PEG (not shown) did not show this shift in retention time. This demonstrates that folate was indeed covalently bound to the PEG chains.

3.3. *Size and charge measurements of the (un)coated polyplexes*

Dynamic light scattering experiments showed that the poly(DMAEMA-*co*-BA)phosphazene is capable to condense plasmid DNA, yielding polyplexes with a size of around 80 nm and a ζ-potential of +22 mV (Fig. 6) at polymer/plasmid ratios (w/w) higher than 6 (N/P = 18). This figure also shows that neutral aggregates were formed between polymer/plasmid ratio of 1.5:1 and 3:1 whereas negatively charged polyplexes with a size of 90 nm were detected at a polymer/plasmid ratio of 0.75:1. This behavior has been observed before for other polymer-based polyplexes (Jiang et al., 2006) and also

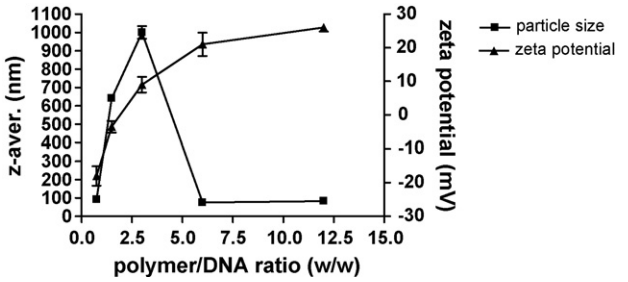

Fig. 6 – Particle diameter and ζ-potential of the poly(DMAEA-*co*-BA)phosphazene polyplexes at different polymer/DNA ratios (w/w) in HEPES buffer (5.0 mM, pH 7.4).

for poly(DMAE)phosphazene (Luten et al., 2003). The addition of NaCl (final concentration 150 mM) to the polyplexes caused a gradual increase in size (from 80 to 170 nm in 10 h). This has been observed before for poly(DMAE)phosphazene and also for other polymers (Luten et al., 2006; Ogris et al., 1998). The surface grafting of PEG to the polyplexes affected both size and surface charge (Fig. 7). The polyplexes were made at a polymer/DNA ratio of 6 (w/w) and after 30 min PEG-NHS or folate-PEG-NHS is added followed by 15 min of incubation. In contrast to naked polyplexes, the PEGylated polyplexes were stable in 150 mM NaCl for more than 4 h. In line with observation for othe pegylated polyplexes (Verbaan et al., 2004; van Steenis et al., 2003), a small increase in size was observed (from to 80 nm (no PEG) to 140 nm at a PEG/primary amine ratio of 6, Fig. 7) with increasing extent of PEG grafting. Zeta potential measurements (Fig. 7) showed that PEG grafting resulted in a drop of the surface charge from +22 to 6 mV, indicating effective shielding of the surface charge of the polyplexes by the PEG chains.

Conjugation of PEG-folate to the polyplexes caused even more pronounced effects (Fig. 8). The size of the targeted polyplexes increased from 80 nm (no PEG) to 160 nm at PEG/primary amine ratio of 6. At the PEG/primary amine ratio of 1.5 and higher, the surface charge of these polyplexes was neutral to slightly negative as has been observed before for folate targeted systems (van Steenis et al., 2003) and is attributed to negatively charged carboxylic acid group of the folate ligand.

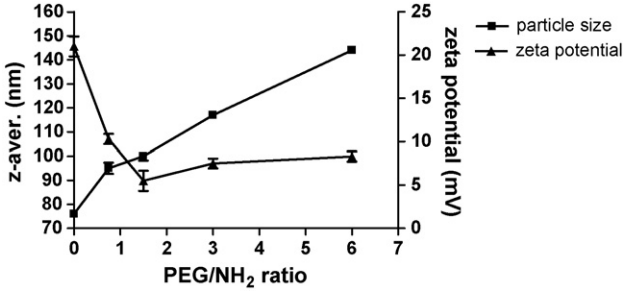

Fig. 7 – Particle diameter and ζ-potential of the post-PEGylated poly(DMAEA-*co*-BA)phosphazene polyplexes at a polymer/DNA ratio of 6 (w/w) and at different PEG/NH_2 ratios in HEPES buffer (5.0 mM, pH 7.4).

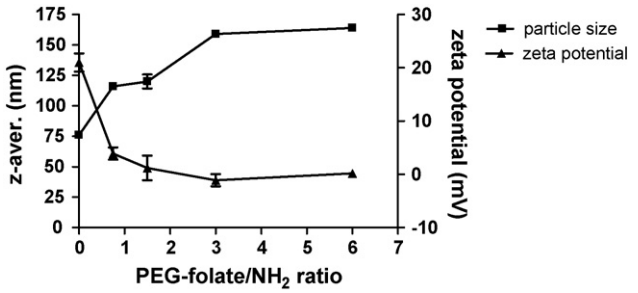

Fig. 8 – Particle diameter and ζ-potential of the targeted poly(DMAEA-co-BA)phosphazene polyplexes at a polymer/DNA ratio of 6 (w/w) and at different PEG/NH_2 ratios in HEPES buffer (5.0 mM, pH 7.4).

3.4. *In vitro transfection studies with (un)coated poly(DMAEA-co-BA)phosphazene-based polyplexes*

The effect of PEGylation with and without folate on the transfection activity of poly(DMAEA-co-BA)phosphazene-based polyplexes was studied using OVCAR-3 cells. Ovarium carcinoma cells are known to have a high expression of folate receptors (Corona et al., 1998).

The addition of the endosomal membrane disrupting INF-7 peptide to the polyplex dispersion, increased the transfection activity with a factor 10–15 for the non-coated as well as the coated poly(DMAEA-co-BA)phosphazene-based polyplexes. Previously we found that, poly(DMAEA)phosphazene-based polyplexes did not benefit from the presence of INF-7 in the transfection of COS-7 cells (Luten et al., 2003). Perhaps either polyplexes are taken up via different routes by COS-7 and OVCAR-3 cells or poly(DMAEA-co-BA)phosphazene- and poly(DMAEA)phosphazene-polyplexes are taken up via different pathways (Wong et al., 2007). It is also possible that the molecular weight of the polymer plays an important role on the transfection properties of poly(DMAEA)phosphazene polyplexes. A recent study (de Wolf et al., 2007a) revealed that the polymer molecular weight not only affects the transfection efficiency of the polyplexes, but also polyplex formation and cytotoxicity. The use of poly(DMAEA-co-BA)phosphazene with a higher molecular weight could lead to better transfection properties, but this needs further investigation.

The effect of PEGylation of the poly(DMAEA-co-BA)phosphazene polyplexes on their transfection activity can be seen in Fig. 9. The activities of polyplexes based on p(DMAEMA) (N/P 5, 42 mU/well) and BPEI (N/P 6, 62 mU/well) were used as a reference. The non-PEGylated polyplexes gave an expression of 45 mU of β-galactosidase per well and were twice as active as the PEG shielded polyplexes. This is expected since shielding of the polyplexes by PEGylation reduces the non-specific binding of the polyplexes and subsequent internalization by the OVCAR-3 cells (Verbaan et al., 2004; van Steenis et al., 2003).

The transfection levels of the folate targeted system were 60 mU of β-galactosidase per well and were even slightly higher than the transfection levels obtained for the uncoated polyplexes. The transfection activity of the polyplexes in the presence of an excess of folic acid (1.0 mM) was also studied (Fig. 9). It was found that the transfection levels of the uncoated polyplexes and for the PEGylated polyplexes were unaffected and remained at the levels obtained without folic acid incubation. Importantly, the transfection activity of the folate targeted polyplexes decreased from 60 to 25 mU β-galactosidase per well, a level comparable to that of the PEGylated polyplexes. These results indicate that the increase in transfection activity of the folate polyplexes is caused by binding of the polyplexes to the folate receptor followed by internalization (folate receptor endocytosis) and eventually to gene expression (Sabharanjak and Mayor, 2004; Lee and Low, 1994).

The relative cell viability of cells incubated with the (un)coated polyplexes is shown in Fig. 10. This figure shows that the uncoated polyplexes displayed a high cell viability (0.8) at a polymer/DNA ratio of 6 and conjugation with PEG or PEG-folate does not alter the cell viability of the gene carrier. For other polymers an increase of cell viability has been observed when polyplexes were coated, but probably because of the low toxicity of the uncoated polyphosphazene polyplexes we do not observe this effect (Lee and Kim, 2005; van Steenis et al., 2003).

3.5. *Confocal laser scanning microscopy*

The cellular association and internalization of the polyplexes was studied using CLSM and fluorescently labeled plasmid. OVCAR-3 cells were incubated with different polyplexes, either uncoated, PEGylated or folate targeted, with a polyphosphazene/plasmid ratio of 6:1 for 1 h at 37 °C. The results are shown in Fig. 11. Fluorescence is observed at the cell membrane as well as in the cytosol of the cells when

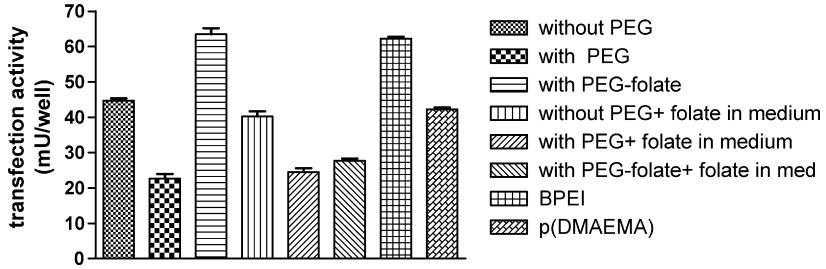

Fig. 9 – Transfection efficiencies of polyplexes, post-PEGylated with NHS-PEG or NHS-PEG-folate at 1.5 PEG:NH_2 ratio, at a polymer/DNA ratio of 6:1 (w/w), in medium, in folate-rich medium (1 mM). Efficiencies were determined by measuring the β-galactosidase activity. Polyplexes based on p(DMAEMA) (N/P 5) and BPEI (N/P 6) were used as a reference.

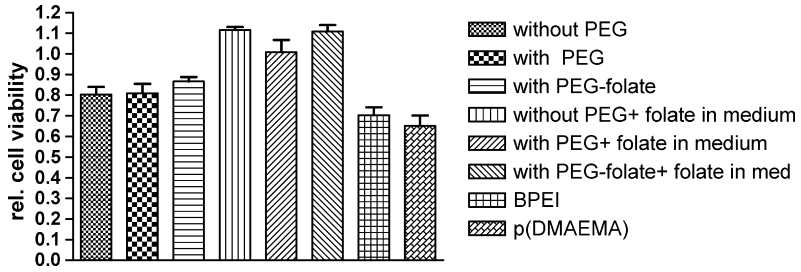

Fig. 10 – Cell viabilities after treatment with polyplexes, post-PEGylated with NHS-PEG or NHS-PEG-folate at 1.5 PEG:NH_2 ratio, at a polymer/DNA ratio of 6:1 (w/w), in medium, in folate-rich medium (1 mM). Polyplexes based on p(DMAEMA) (N/P 5) and BPEI (N/P 6) were used as a reference.

they were incubated with uncoated polyplexes, indicating cell association and cell internalization (Fig. 11A). This is in line with previous papers in which it is shown that positively charged polyplexes are able to bind to and be taken up by cells (Zuidam et al., 2000). For the PEGylated phosphazene polyplexes less intense fluorescence was observed both at the cell membrane and in the cytosol (Fig. 11B) which points to lower non-specific interaction between the PEGylated polyplexes and cells as compared to non-coated particles. Lower binding of the polyplexes to the cell lead to decrease in cellular uptake of the polyplexes and a lower transfection activity, in line with the observations reported in Fig. 9. Incubation of cells with folate-PEG polyplexes (Fig. 11C) caused a more intense fluorescence both at the cell membrane and in the cell as compared to the PEGylated polyplexes, suggesting more cell association and internalization of the targeted polyplexes. PEGylation with folate-PEG restores the cellular uptake of the polyplexes, leading to higher transfection levels, as observed in the transfection experiments.

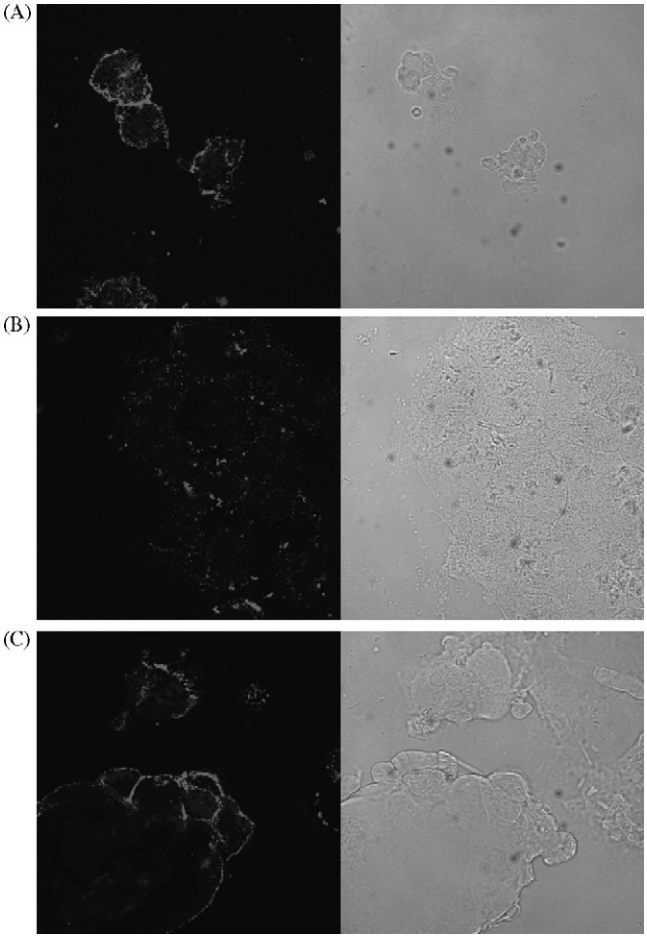

Fig. 11 – CLSM-study of OVCAR-3 cells upon incubation with poly(DMAEA-co-BA)phosphazene/DNA 6:1 polyplexes for one hour at 37 °C (A), post-PEGylated poly(DMAEA-co-BA)phosphazene/DNA 6:1 polyplexes (B) and post-PEGylated with folate-PEG poly(DMAEA-co-BA)phosphazene/DNA 6:1 polyplexes (C). The pictures to the right are the direct transmitted light pictures taken simultaneously with the CLSM-pictures. The plasmid DNA was labeled with Cy5.

3.6. *Polyplex-induced aggregation of erythrocytes*

Upon intravenous injection of polyplexes, interactions between polyplexes and blood components will occur (Verbaan et al., 2004; Dash et al., 1999; Moreau et al., 2002). To study whether PEGylation decreases adverse interactions between the polyplexes and erythrocytes, the different polyplexes (polymer/plasmid ratio was 6), either uncoated, PEGylated, or with PEG-folate, were incubated with erythrocytes and the aggregation was monitored visually. Fig. 12 shows the haemaglutinating properties of the polyplexes when they were incubated with erythrocytes. As a control pDMAEMA-based polyplexes (polymer/DNA ratio 3:1) were used. The pDMAEMA-based polyplexes possessed the strongest erythrocyte aggregating activity as reflected by the lowest polymer concentration at which erythrocyte aggregation did not occur (0.04 μg/mL). Also the uncoated polyphosphazene-based polyplexes displayed a relatively high erythrocyte aggregating activity (0.09 μg/mL). When the polyphosphazene-based polyplexes were post-PEGylated with PEG or with PEG-folate (1.5 equivalents to N) erythrocyte aggregation occurred at a 15 times higher polymer concentration as compared to the uncoated polyphosphazene-based polyplexes. This shows that PEG indeed effectively shields the positive charge of the polyplexes thereby reducing a specific cellular interactions.

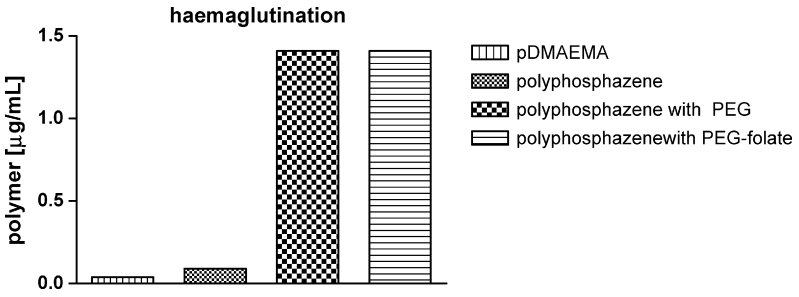

Fig. 12 – Erythrocyte aggregation by different polyplexes. Haemaglutination is expressed as the highest polymer concentration at which aggregation was not observed ($n = 2$).

4. Conclusion

We synthesized a new cationic co-polymer polyphosphazene with pendant primary and tertiary amine side groups. Polyplexes based on this polymer could be post-PEGylation with PEG and PEG-folate, leading to almost neutral nanoparticles. We showed that the transfection activity of PEG-folate polyplexes was folate receptor-specific. Erythrocyte aggregation was suppressed when the polyplexes were PEGylated. Future work will focus on *in vivo* application of this new polymer.

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