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RESEARCH PAPER

Amine-Modified Poly(Vinyl Alcohol)s as Non-viral Vectors for siRNA Delivery: Effects of the Degree of Amine Substitution on Physicochemical Properties and Knockdown Efficiency

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ABSTRACT

Purpose The objective of this study was to investigate how the degree of amine substitution of amine-modified poly(vinyl alcohol) (PVA) affects complexation of siRNA, protection of siRNA against degrading enzymes, intracellular uptake and gene silencing.

Methods A series of DEAPA-PVA polymers with increasing amine density was synthesized by modifying the hydroxyl groups in the PVA backbone with diethylamino propylamine groups using CDI chemistry. These polymers were characterized with regard to their ability to complex and protect siRNA against RNase. Finally, their potential to mediate intracellular uptake and gene silencing in SKOV-luc cells was investigated.

Results A good correlation between amine density and siRNA complexation as well as protection of siRNA against RNase was found. Consisting solely of tertiary amines, this class of polymer was able to mediate efficient gene silencing when approximately 30% of the hydroxyl groups in the PVA backbone were modified with diethylamino propylamine groups. Polymers with a lower amine density (up to 23%) were inefficient in gene silencing, while increasing the amine density to 48% led to non-specific knockdown effects.

Conclusion DEAPA-PVA polymers were shown to mediate efficient gene silencing and offer a promising platform for further structural modifications.

KEY WORDS amine density · gene silencing · non-viral vectors · polycation · siRNA

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INTRODUCTION

The clinical application of nucleic acid therapy depends critically on the development of safe and effective carriers for intracellular delivery of genetic material. Non-viral vectors, such as polymers, show promise as siRNA delivery vehicles due to their potential for structural modifications. Different classes of polymers with amine functionalities, such as polyethylenimine, chitosan, poly(L-lysine) and PAMAM dendrimers, have not only been used for DNA but also siRNA delivery (1,2). Modifications of the polymeric backbone were often required to increase and optimize gene silencing efficiency. siRNA delivery has benefited from the advances made in DNA delivery using synthetic vectors; however, a direct transfer of the knowledge gained in the longer-studied DNA delivery field to siRNA applications is only possible to a certain extent. The large difference in sizes of siRNA (21–23 base pairs) and pDNA (up to several kilo base pairs) and the different cellular locations of the site of action (cytosol *versus* nucleus) are some of the many reasons why certain DNA technologies may not be suitable for gene silencing (3). A variety of cationic polymers has been studied as siRNA carriers, most of them are based on self-assembly via electrostatic interactions. These studies demonstrated the importance of finding the optimal balance between sufficient siRNA binding and protection, as well as unpackaging of siRNA in the cytosolic environment combined with low toxicity. In the case of cyclodextrin-containing polycations (CDP) developed by Davis and coworkers, the distance between the cyclodextrin and the amidine charge centers was varied to achieve appropriate nucleic acid binding affinity and delivery (4,5). Gabrielson *et al.* showed that acetylation of PEI increased gene delivery activity by 20- to 60-fold by weakening the electrostatic binding to the nucleic acid compared to unmodified PEI (6). In line with these observations,

Yezhelyev *et al.* controlled the affinity to the siRNA by tuning the ratio between carboxylic acid and tertiary amines on the surface of the quantum dots. They showed that carboxylic anions weakened siRNA binding to the carrier, thereby enabling its release in the cytosol (7). As polymers containing primary and secondary amines have been shown to condense and complex siRNA to a high extent, but also caused higher toxicity (8), our objective was to investigate whether tertiary amines alone are able to complex and protect siRNA in a way that would be sufficient for effective gene silencing. Kong *et al.* recently demonstrated that a ternary complex consisting of the homopolymer dimethylaminoethyl methacrylate (DMAEMA) containing solely tertiary amines, its PEG-derivative, and siRNA is able to decrease the production of VEGF in PC-3 cells by gene silencing (9). Our approach to contribute to the understanding of the structure-activity relationship between a polymer structure and its performance in siRNA delivery is based on a series of amine-modified poly(vinyl alcohol)s (PVA). PVA with a molecular weight of 15,000 g/mol was chosen as a backbone, as it is water soluble, is considered to be biocompatible, and can be renally excreted from the body (10,11). To examine the effect of the degree of amine substitution on physicochemical characteristics and biological activity of polyplexes, a panel of 5 polymers was synthesized. These polymers were thoroughly investigated with regard to siRNA complexation efficiency, protection against RNase, intracellular uptake and knock-down efficiency in SKOV-luc cells.

MATERIALS AND METHODS

Materials

3-(Diethylamino)propylamine (purum, ≥99%) was purchased from Sigma Aldrich (Germany). Poly(vinyl alcohol) (Mowiol 3-85) was obtained from Clariant (Germany). The anti-luciferase siRNA: 5′-GAUUAUGUCCGGUUAU GUA-3′ (cat. No. D-002050-01-20) and siCONTROL Non-Targeting siRNA #3 (cat. no. D-001210-03-20) were purchased from Dharmacon (Lafayette, CO, USA). Fluorescein-Luc siRNA was purchased from MWG Biotech (Germany). Lipofectamine 2000 was obtained from Invitrogen (CA, USA). The SKOV-luc cells were a kind gift from Dr. Aigner (Philipps-Universität Marburg, Germany). RNase A was purchased from Sigma Aldrich (Germany).

Synthesis of Amine-Modified PVAs

Amine-modified poly(vinyl alcohol)s were synthesized as previously described (12). Briefly, 3-diethylamino-1-propylamine (DEAPA) was activated with carbonyldiimidazole. In a polymer-analogous reaction, these amine groups were covalently coupled to the polymer backbone poly(vinyl alcohol) (PVA). Polymers with the source-based IUPAC nomenclature of poly-(vinyl 3-(diethylamino) 1-propyl carbamate-co-vinyl acetate-co-vinyl alcohol) were obtained. As an abbreviation, the short form of the amine and poly(vinyl alcohol) will be used, followed by a number in brackets: DEAPA-PVA (z), with z displaying the percentage of modified vinyl alcohol monomers in the chain (degree of amine substitution, DS). For this study, the following DEAPA-PVA polymers were synthesized: DEAPA-PVA (19), DEAPA-PVA (23), DEAPA-PVA (32), DEAPA-PVA (43) and DEAPA-PVA (48).

Polymer Characterization

Polymers were characterized by ^{1}H- and ^{13}C- NMR spectroscopy. Data were collected by a JEOL Eclipse 500 and a JEOL GX 400 D. Ca. Forty to fifty mg of the polymers were dissolved in DMSO-d_6. ^{1}H-NMR was performed with 64 scans, and ^{13}C-NMR was performed with 4,096 scans at 60°C. The degree of amine substitution was determined by calculating the ratio between the integral of the CH_3 end group of the DEAPA (δ=0.95) and the integral of the methylene group of the PVA (δ= 1.87−1.17) as previously described (12,13).

Gel Permeation Chromatography

For the determination of absolute molecular weights and weight distributions, gel-permeation chromatography (GPC), in combination with a multi-angle-laser-light scattering detector (GPC-MALLS), was employed. The measurements were performed using an Optilab DSP together with a PSS SDV linear M (8×300 mm, 5 µm) column with a precolumn of the same type (8×50 mm, 5 µm) at 60°C. The eluent consisted of dimethylacetamide (DMAc) and 2.5 g lithium bromide/L (LiBr). Runs were performed at a flow rate of 0.5 ml min^{-1}. The molecular weights of the samples were determined using the Wyatt software Astra V5.1. To calculate the molecular weights, total mass recovery was used as described previously (12).

Cytotoxicity in SKOV-luc Cells

MTT assay was performed to determine the cytotoxicity of the DEAPA-PVA polymers. SKOV-luc cells were seeded into 96-well plates at a density of 8,000 cells/well. After 24 h, the cell culture medium was replaced with increasing polymer concentration in 100 µl cell culture medium. After 24 h incubation, medium was replaced by DMEM without serum, containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Germany). After 4 h incubation at 37°C, medium was aspirated, and formazane crystals were

dissolved in 200 µl DMSO per well. Measurement was performed using an ELISA reader (Titertek Plus Ms 212, ICN, Eschwege, Germany) at a wavelength of 570 nm and 690 nm. Relative viability was calculated using 0% (wells without cells) and 100% (wells with untreated cells) controls. Data are presented as a mean of four measurements. IC_{50} was calculated using the Boltzman sigmoidal function from Microcal Origin® v 7.0 (OriginLab, Northampton, USA). Experiments were repeated twice.

Polyplex Formation

All polyplexes were prepared in HEPES buffered glucose (HBG) (5% Glucose buffered with 10 mM HEPES, pH 7.4 freshly before use). Briefly, the polymer solutions were added to the siRNA solutions in equal volumes (100 µl each), mixed by vigorous pipetting and incubated for 20 min at room temperature prior to use. The siRNA concentration in the resulting polyplex solutions was 12.5 µg siRNA/ml. To maintain NP ratios between 7 and 30, the polymer concentrations were adjusted to the amount of siRNA. Lipofectamine 2000 was used as a positive control and prepared according to the manufacturer's protocol. Briefly, 1 µl of Lipofectamine 2000 was diluted with HBG to 10 µl, mixed with 10 µl of HBG containing 0.25 µg siRNA and incubated for 20 min prior to use.

Hydrodynamic Diameter and Zeta Potential

The hydrodynamic diameters of the polyplexes were determined by photon correlation spectroscopy (PCS) using the Zetasizer, Nano ZS, Malvern Instruments (Herrenberg, Germany). The z-average (Z_{ave}) diameters of five measurements were reported. For determination of the zeta potential, the polyplex solution was further diluted in distilled water. For data analysis, the viscosity (0.8905 mPas) and refractive index (1.333) of distilled water at 25°C were used. All measurements are given as mean values of three independent runs each performed in triplicate.

Atomic Force Microscopy

For atomic force microscopy (AFM) a 10 µl droplet of the polyplex solution was dispersed on a glass slide, and polyplexes were allowed to adhere for 5 min. After this time, the supernatant was removed, and the sample was dried in a dry nitrogen gas flow. Samples were investigated within 2 h of preparation. AFM was performed on a JPK NanoWizard (JPK Instruments, Berlin, Germany). Silicon nitride tips attached to cantilevers of a length of 230 µm and a resonance frequency of about 160 kHz were used (NSC16 AlBS, Micromasch, Estonia). AFM was performed in intermittent contact mode to minimize forces applied to the sample. The amplitude images shown were recorded at a scan frequency of 1 Hz.

Knockdown Efficiency of Polyplexes

Human ovarian carcinoma cells, SKOV-luc cells, were stably transfected to express firefly luciferase and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of 8,000/well. Twenty µl of polyplexes or lipofectamine/siRNA formulation, corresponding to 0.25 µg of siRNA, were added to each well containing 100 µl of medium supplemented with fetal calf serum. After 4 h incubation, medium was replaced, and the cells were allowed to grow for 44 h. In addition to anti-luc siRNA, non-specific siRNA was used to determine unspecific gene silencing by cytotoxic effects or off-target effects.

Luciferase gene silencing activity was measured according to the protocol provided by Promega (Madison, WI, USA). Briefly, cells were washed with PBS and lysed in 100 µl cell culture lysis buffer for 15 min. Luciferase light units were quantified by automatic injection of 50 µl freshly prepared luciferase assay buffer containing 10 mM luciferin, to a 20 µl aliquot of the cell lysate. The relative light units (RLU) were measured with a plate luminometer (LumiSTAR Optima, BMG Labtech GMBH, Offenburg, Germany). Data were expressed as percentage of control (untreated cells). Luciferase expression of untreated non-transfected cells were set as 100%. All experiments were performed in quadruplicate and are representative of three independent experiments.

Complexation Efficiency by Ethidium Bromide Exclusion Assay

Complexation of siRNA was evaluated in HBG using an ethidium bromide exclusion assay. Briefly, 0.4 µg siRNA was complexed with increasing amounts of polymer to obtain the following NP ratios: 1, 3, 5, 7, 10, 15, 20 and 30. After 20 min incubation at RT, 20 µl of a 0.0125 mg/ml ethidium bromide solution was added, and solutions were mixed intensively. 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. Uncomplexed siRNA was quantified by a calibration curve ranging from 0.0156 µg to 0.75 µg siRNA. Results are given as means of triplicate measurements ± sd and are representative of three independent experiments.

siRNA Protection Against RNase

For siRNA stability studies, DEAPA-PVA/siRNA polyplexes and PEI 25 kDa/siRNA polyplexes were incubated with increasing amounts of RNase A. Polyplexes were prepared at NP of 15 in HBG. Aliquots of 40 µl polyplexes containing 0.5 µg of siRNA were incubated with 0.15, 0.25, 0.5, 1.5, 2, 4 and 8 mIU of RNase in 2 µl HBG (pH 7.4) for 30 min at 37°C. To inactivate the RNase, the samples were incubated for a further 30 min at 70°C. Samples were incubated with 2 IU of heparin to release the siRNA from the polyplexes. The resulting mixtures were applied to a 20% polyacrylamide gel, and electrophoresis was carried out at 100 V for 1 h. The gel was stained for 5 min in ethidium bromide and visualized using a transilluminometer (Biometra, Göttingen, Germany). The intensity of intact recovered siRNA from the polyplexes after treatment with RNase was quantified by Image J. The percentage of siRNA dissociated from RNase-treated polyplexes was obtained by normalizing against the siRNA band from untreated polyplexes on the same gel. Results are given as mean values ± sd of three independent runs.

Uptake Studies in SKOV-luc Cells by FACS

For uptake studies, SKOV-luc cells were seeded on 48-well plates at a density of 2.5×10^4 cells/well 24 h prior to the experiment. For polyplex formation fluorescein-labeled siRNA was used. Cells were incubated with 50 µl of the polyplex formulations at different time points (0.5 h, 1 h, 2 h, 3 h and 4 h) at 37°C. After washing the cells with PBS, extra-cellular fluorescence was quenched by incubation with Trypan blue 0.4% for 5 min, followed by washing with PBS again. Cells were detached by trypsinization and were resuspended in a 1:1 mixture of FACSFlow (BD Biosciences, San Jose, CA) and 4% paraformaldehyde in PBS for cell fixation. Amount of fluorescein-labeled siRNA taken up by SKOV-luc cells was measured by FACScan (BD Biosciences, San Jose, CA) with excitation at 488 nm and emission filter set to 530/30 band pass. Five-thousand cells were counted for each sample. Experiments were performed in triplicate and are representative of three independent experiments. Data acquisition and analysis were performed using CellQuest Pro (BD Biosciences, San Jose, CA) and FCS Express V3.00 (DeNovo Software, Thornhill, Canada).

Buffer Capacity

The buffering capacities of the DEAPA-PVA polymers compared to PEI 25 kDa were determined by acid-base titration. Prior to titration, 10 ml of a 2 mg/ml polymer solution or the amount of PEI equivalent to the mol of amines contained in 20 mg of DEAPA-PVA polymer was adjusted to pH 11.0 by 0.1 M NaOH. The titration was performed using the titrator DL55 (Mettler-Toledo, Giessen, Germany) equipped with a 10 ml burette and a titration stand. The polymer solutions were titrated from pH 11 to pH 2 with 0.1 M HCl. Data were recorded with the software LabX light V. 2.1 (Mettler-Toledo, Giessen, Germany). Experiments were performed in triplicate.

Membrane Interactions Using Liposomes as a Membrane Model

Negatively charged liposomes were prepared by dissolving dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) at a molar ratio of 9:1 in a 20 ml mixture of chloroform methanol (7:3 volume fractions). To obtain a thin lipid film, the solvent mixture was removed under reduced pressure at 60°C using a rotavapor and was dried for further 60 min under vacuum (14). The film was rehydrated with 5 ml of carboxyfluorescein solution (50 µg/ml, pH 7.4), and liposomes were prepared by incubation in an ultrasonic bath for 10 min at 60°C. Dispersed liposomes were stored at 4°C for 1 h, then extruded through a 400 nm polycarbonate filter (Lipofast, Avestin, Ottawa, Canada) 21 times at 60°C. Non-encapsulated carboxyfluorescein was separated from the liposomes by centrifugation and resuspended five times. Polyplexes (150 µl) were incubated with 50 µl liposomes for 4 h at 37°C under constant shaking. Released carboxyfluorescein caused by membrane interactions was separated by centrifugation for 60 min at 6,000 g. PBS and 1% Triton X-100 in PBS were used as controls for 0% lysis and 100% lysis, respectively. Released carboxyfluorescein was calculated by a calibration curve ranging from 5 ng/ml to 50 ng/ml. Results are given as means of triplicate measurements ± sd and repeated twice.

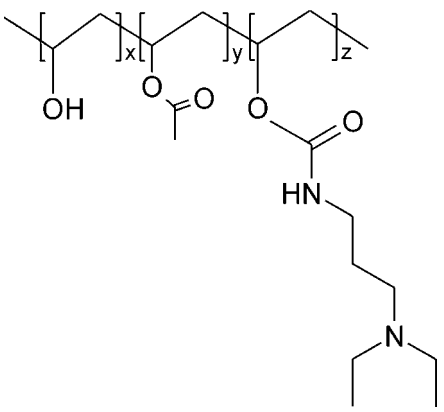

Fig. 1 Chemical structure of DEAPA-PVA polymers. x, y and z stand for the repeat units of the PVA-DEAPA polymer, where x is the number of unmodified monomers in the backbone, y the number of acetate-substituted monomers and z is the number of monomers in the backbone carrying amine substitutions.

Table I DEAPA-PVA Polymers: Synthesis and Molar Mass

Polymer	Feed ratio[a]	Amine substitution[b]	M_n[c] g/mol	M_n[d] g/mol	M_w[d] g/mol	M_w[d] / M_n[d]
DEAPA-PVA(19)	2.00/4.10/0.23/4	19	23,590	nd	nd	nd
DEAPA-PVA(23)	2.00/4.62/0.26/4	23	25,620	27,630	43,820	1.586
DEAPA-PVA(32)	2.00/5.16/0.29/4	32	29,840	32,700	54,280	1.659
DEAPA-PVA(43)	2.00/11.15/0.67/4	43	34,530	38,000	73,770	1.941
DEAPA-PVA(48)	2.00/18.59/1.11/4	48	36,870	45,150	82,010	1.816
Unmodified PVA			15,000	14,780	22,340	1.511

[a] feed (mass/g): m(PVA)/m(amine-Cl)/m(DMPU)/days of reaction; [b] degree of amine substitution (%); [c] calculated by ^{1}H NMR depending on the degree of polymerization of PVA ($P = 300$); [d] measured by GPC-MALLS

Statistical Analysis

Significance between the mean values was calculated using unpaired Student's *t*-tests or one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. Statistical differences are presented at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS

Synthesis and Characterization of DEAPA-PVA Polymers by NMR and GPC

A series of DEAPA-PVA polymers with different degrees of amine substitution was successfully synthesized (Fig. 1). Based on the CDI chemistry, Diethylaminopropylamine was covalently coupled to PVA in different degrees of substitution ranging from 55 (DS = 19%) to 140 (DS = 48%) modified vinyl alcohol monomers in the chain. PVA with a molar mass of 15,000 g/mol was used. To determine the degree of substitution, the signal of PVA at $\delta = 1.87$–1.17 ppm (methylene protons) was related to the signal of the amine substitution at $\delta = 0.95$ ppm, caused by the DEAPA (13). The structures were confirmed by NMR and GPC measurements, shown in Table I.

Cytotoxicity of DEAPA-PVA Polymers

Cytotoxicity of the DEAPA-PVA polymers was evaluated by an MTT-assay. As depicted in Fig. 2, cytotoxicity of all DEAPA-PVA polymers tested increased with increasing amine density DEAPA-PVA(19) < DEAPA-PVA(23) < DEAPA-PVA(32) < DEAPA-PVA(43) < DEAPA-PVA(48). DEAPA-PVA(32), which showed the highest knockdown efficiency was two-fold less toxic than DEAPA-PVA(43) and DEAPA-PVA(48). PEI 25 kDa served as a comparison.

Hydrodynamic Diameter and Zeta Potential

Hydrodynamic diameters and zeta potentials of DEAPA-PVA/siRNA and PEI/siRNA polyplexes were determined at NP ratios from 7 to 20 using dynamic light scattering (DLS). The polyplexes were prepared in HEPES buffered

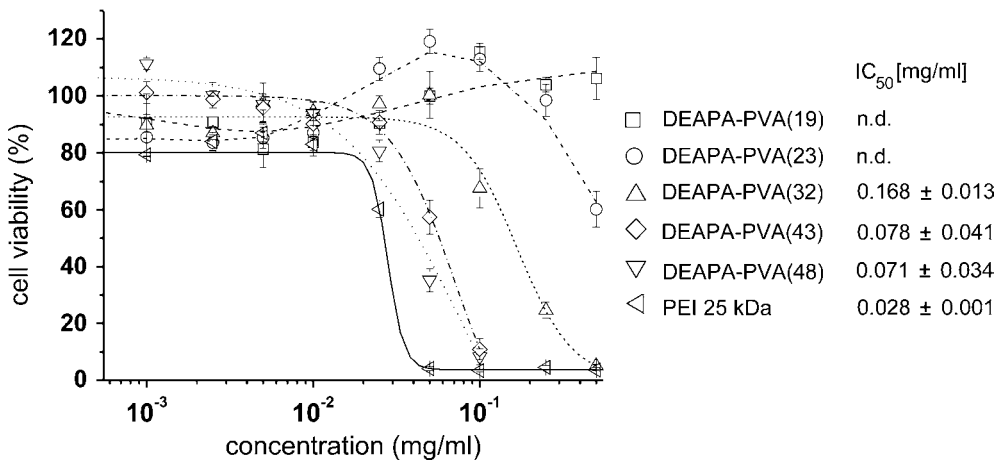

Fig. 2 Toxicity profiles and IC_{50} values of DEAPA-PVA polymers. PEI 25 kDa served as comparison.

glucose (HBG). As displayed in Fig. 3A, hydrodynamic diameters (Z_{ave} values) of DEAPA-PVA/siRNA polyplexes were between 400–600 nm with a PDI in the range of 0.35 and 0.5, indicating a broad size distribution of the polyplexes. Within the DEAPA-PVA polymer series, there was no decrease in size with increasing NP ratio or increasing amine-density. The values of the surface charges determined by the zeta potential increased with higher amine density (Fig. 3B), starting from +17 mV for DEAPA-PVA (19) to +45 mV for the DEAPA-PVA (48) polymer. PEI 25 kDa formed relatively large polyplexes with siRNA.

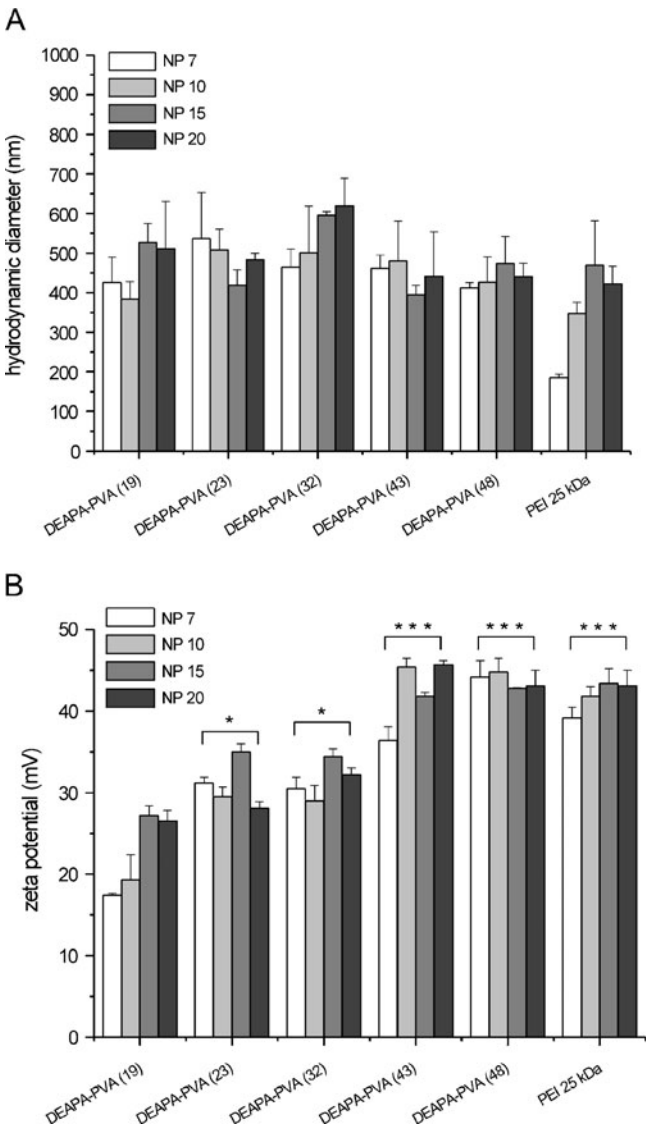

Fig. 3 (**A**) Hydrodynamic diameter of DEAPA-PVA/siRNA and PEI 25 kDa/siRNA polyplexes at different NP ratios, (**B**) Zeta potential of DEAPA-PVA/siRNA and PEI 25 kDa/siRNA polyplexes at different NP ratios. No statistical differences were found between the sizes of all DEAPA-PVA polymers. The zeta potential values of DEAPA-PVA(23) and DEAPA-PVA(32) (*$p < 0.05$), DEAPA-PVA(43) and DEAPA-PVA(48) (***$p < 0.001$), are all significantly different from DEAPA-PVA(19).

The hydrodynamic diameters of PEI 25 kDa/siRNA polyplexes were around 170 and 350 nm for NP ratios 7 and 10 and increased to 450 nm at higher NP ratios. The large size was most likely due to aggregation shown by the high polydispersity index of ~0.46±0.03. Zeta potential values of PEI 25 kDa were ~ +43 mV for all NP ratios and comparable to that of DEAPA-PVA(43) and DEAPA-PVA (48). The high zeta potential of the DEAPA-PVA(43) and DEAPA-PVA(48) polymers is most likely due to tertiary amines with a pK_a value of 9.8–10.2 (see also Fig. 8). At physiological pH (pH 7.4), these tertiary amines were completely protonated and positively charged. In contrast to the DEAPA-PVA polymers, PEI 25 kDa not only contains tertiary amines (26%), but also primary (32%) and secondary (42%) amines. Due to the pK_a values, which ranged from 6 to 9 depending on the type of amine, only partial protonation (~20%) of the amines occurred at pH 7.4 (15).

Atomic Force Microscopy

Atomic force microscopy (AFM) was used to gain more detailed information about the morphology and heterogeneity of the polyplexes. Representative images of DEAPA-PVA(32) and DEAPA-PVA(48) were shown. In accordance with the high polydispersity index measured by DLS, DEAPA-PVA polymers formed polyplexes with a broad distribution in particle size. Small polyplexes with a diameter around 100–125 nm (Fig. 4 A&B) and larger aggregates of approximately ~240–350 nm (Fig. 4 C&D) were observed. The difference between AFM and DLS data in particle sizes can be attributed to the fact that different particle-sizing techniques can lead to different size measurements, as each technique is sensitive to different properties of the particles (i.e. polydispersity, scattering intensity, shape, surface structure, etc.) (16). In the case of DLS, small polyplexes (weakly scattering) could have been masked by the higher scattering of DEAPA-PVA polyplex aggregates, leading to an overestimation of the hydrodynamic diameter (17). Additionally, for AFM measurements, the polyplex solutions were dried, which involved removing them from their aqueous environment as well as detaching them from their hydration shell, thus leading to smaller sample sizes (18).

Complexation Efficiency

Complexation of siRNA is one of the key factors for efficient gene silencing and is typically dependent on the chemical structure of each polymer. The amine density of a polymer plays the predominant role in compaction of siRNA. Too tight binding, as in the case of PEI 25 kDa, could lead to low knockdown efficiency due to insufficient

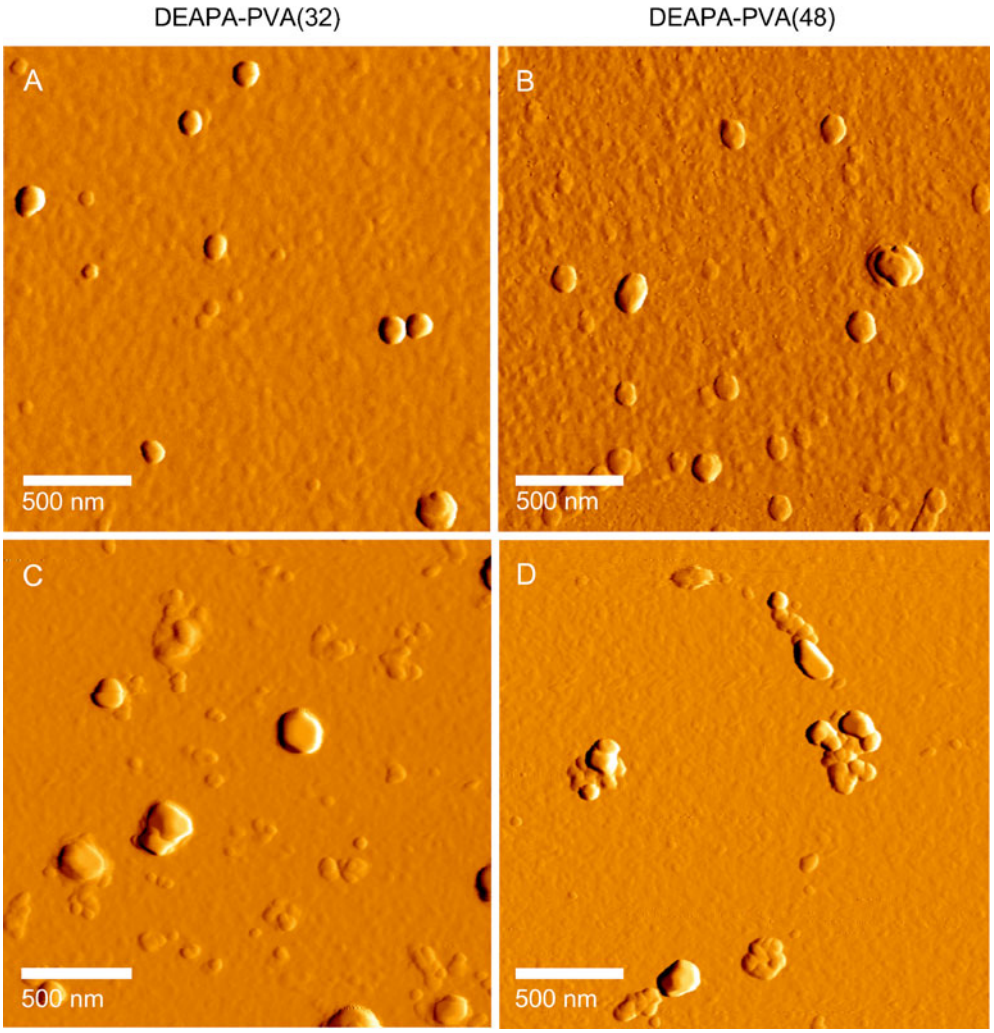

Fig. 4 AFM topographical images of siRNA polyplexes formed with DEAPA-PVA(32) (**A**,**C**) and DEAPA-PVA(48) (**B**,**D**) at NP 15. Representative images are shown.

cytosolic release of the RNA (19), while instable polyplexes would not provide adequate protection against degrading enzymes such as RNase (3). The formation of siRNA polyplexes at different NP ratios (0–20) was investigated by ethidium bromide fluorescence measurements. Fig. 5A displays the siRNA complexation efficiency of the DEAPA-PVA polymers in comparison to PEI 25 kDa. The two polymers with the lowest amine density, DEAPA-PVA (19) and DEAPA-PVA (23), showed very little siRNA complexation efficiency. At an NP ratio of 20, 64% of the siRNA remained free or insufficiently bound. With DEAPA-PVA (32), free siRNA could be slightly reduced to 60% at NP ratios between 10 and 20. The two polymers with the highest amine density, DEAPA-PVA (43) and DEAPA-PVA (48), formed relatively stable complexes with siRNA, leading to only 30–40% unbound siRNA. As shown in Fig. 5B a good correlation between amine density and complexation efficiency was found ($R^2 = 0.9712$). To establish the correlation, 15 was chosen as a representative NP ratio. A good correlation between amine density and complexation efficiency of siRNA was also found for NP ratios 3, 7, 10 and 20, with correlation coefficients (R^2 value) in the range of 0.9657–0.9857. As expected, PEI 25 kDa revealed the highest binding affinity to siRNA; a complete complexation could be achieved with an NP ratio of 5.

Polyplex Stability Against RNase

Insufficient protection of siRNA against enzymatic degradation leads to a decrease in gene silencing efficiency and biological activity. In order to investigate the polyplex stability against nuclease digestion, polyplexes were incubated with increasing amounts of RNase A. As displayed in Fig. 6A and B, DEAPA-PVA(19) and DEAPA-PVA(23), the two polymers with the lowest amine density, provided insufficient protection of the siRNA. In line with the incomplete complexation of siRNA, shown in the ethidium bromide assay, incubation with small amounts of RNase led to a rapid decline of intact siRNA. At an RNase concentration of 4 mIU/μg siRNA, 40% was left in the case of DEAPA-PVA(23), while intact siRNA

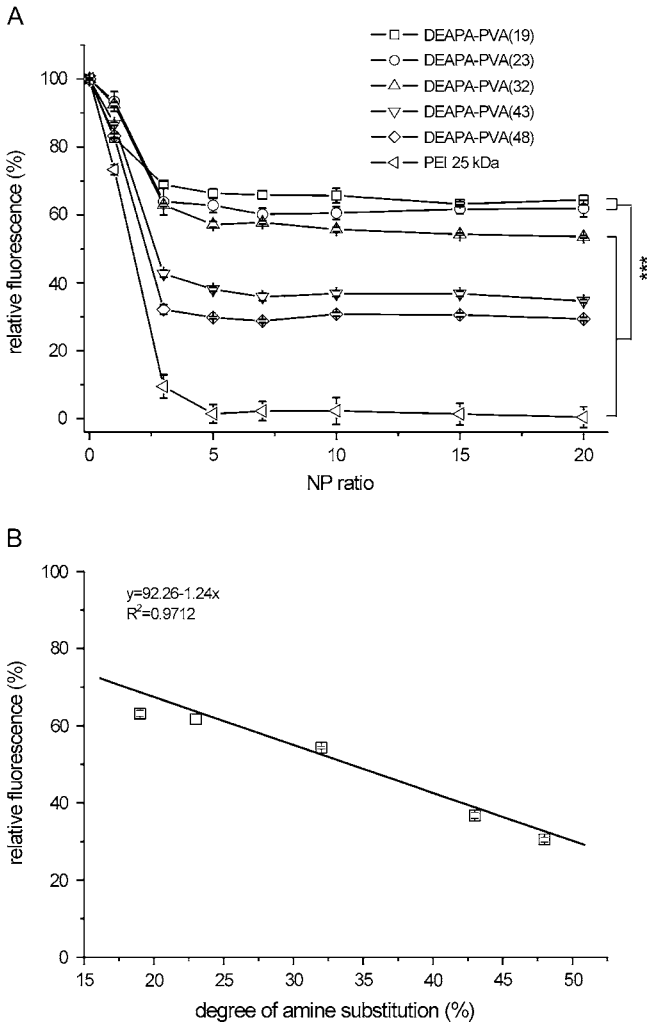

Fig. 5 (**A**) siRNA complexation efficiency of DEAPA-PVA polymers compared to PEI 25 kDa, studied by ethidium bromide exclusion assay. Significant differences in complexation efficiency compared to both polymers with the lowest degree of amine substitution: DEAPA-PVA(19) and DEAPA-PVA(23) are marked with ***$p < 0.001$; DEAPA-PVA(32) *** $p < 0.001$ at NP 10–20; DEAPA-PVA(43), DEAPA-PVA(48) and PEI 25 kDa ***$p < 0.001$ at NP 1-20, (**B**) Correlation between degree of amine substitution and siRNA complexation efficiency. NP 15 was chosen as a representative NP ratio.

was reduced to only 10% when complexed with DEAPA-PVA(19). Complete siRNA degradation occurred with both polymers after exposure to 8 mIU RNase/μg siRNA. In contrast, the DEAPA-PVA polymers with a higher amine density and PEI 25 kDa provided more efficient nucleic acid protection. When DEAPA-PVA(32)/siRNA polyplexes were challenged with 8 mIU RNase/ μg siRNA, 40% of the siRNA remained intact. siRNA protection was increased to 70% in the case of DEAPA-PVA(43), DEAPA(48) and PEI 25 kDa. As displayed in Fig. 6C, a remarkably good correlation ($R^2 = 0{,}9958$) between amine density and the percentage of siRNA remaining could be found.

Uptake Studies in SKOV-luc Cells by FACS

The first barrier for efficient transfection *in vitro* is sufficient uptake of polyplexes into cells by endocytosis. Flow cytometry measurements were performed to determine the amount of siRNA taken up into SKOV-luc cells. As shown in Fig. 7, almost no uptake of siRNA could be observed when complexed with the lowest amine-dense polymer DEAPA-PVA (19). This is most likely due to the insufficient complexation and protection of siRNA as described above in the ethidium bromide complexation and RNase digestion assay. With DEAPA-PVA (23)/siRNA polyplexes, a slight increase in siRNA uptake was observed. In general, intracellular uptake of siRNA increased with longer incubation time. After 4 h of incubation DEAPA-PVA(32), DEAPA-PVA(43) and DEAPA-PVA(48) mediated a 1.7–2.1-fold increase in siRNA uptake compared to DEAPA-PVA(23) and a 10–13-fold increase in siRNA uptake compared to DEAPA-PVA(19).

Buffer Capacity

For PEI 25 kDa, the endosomal escape via proton-sponge effect has been postulated as the main release mechanism into the cytosol. Its buffer capacity in the pH range from 5 to 7 is proposed to be a key factor for its high transfection efficiency. Causing an increase in osmotic pressure, the endosomal membrane gets disrupted, leading to the release of the polyplexes into the cytosol (20). Therefore, we determined the buffering capacity of the DEAPA-PVA polymers by base-acid titration (Fig. 8A). As there is a pH drop from the endosomes to the lysosomes, the buffering ability of the polymers in the pH-range from 7.4 to 5.0 is of most interest. Unlike PEI 25 kDa, the DEAPA-PVA polymers only possess tertiary amines with a pK_a of ~9.8–10.2. This resulted in a complete protonation at physiological pH 7.4 with negligible buffering capacity in the pH range from pH 7.4 to 5.0, shown by the vertical trend of the titration curve. In Fig. 8B, the titration curves of PEI at equivalent amine mol concentrations to the DEAPA-PVA polymers are shown. The buffer capacity of PEI over a broader pH range (5–10) is governed by the presence and strong interactions of neighbouring tertiary, secondary and primary amines, which displayed pK_a values ranging from 6 to 9 (15,21).

Interaction with Liposomes as a Model for Cell Membranes

The release of polyplexes from the endosomal compartment can occur in different ways. Apart from endosomal escape via "proton-sponge effect," membrane fusion in the case of liposomes or membrane destabilization due to electrostatic interactions of the polyplexes with the cell membranes have been shown to play an important role in

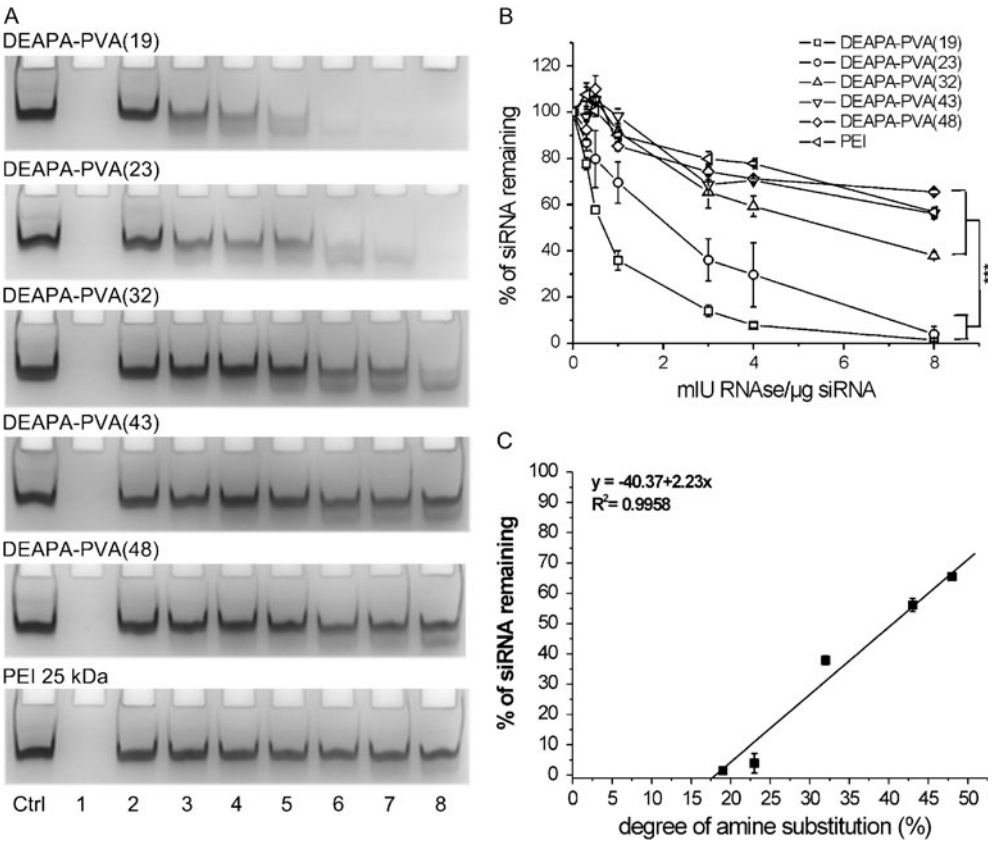

Fig. 6 (**A**) Polyplex stability in presence of RNase A. Polyplexes (NP 15) were challenged with increasing amounts of RNase A for 30 min at 37°C, Ctrl: naked siRNA, lane 1: naked siRNA treated with 0.15 mIU RNase/0.5 μg siRNA, lanes 2–8: polyplexes treated with RNase 0.15, 0.25, 0.5, 1.5, 2, 4 and 8 mIU/0.5 μg siRNA. (**B**) Percentage recovery of intact siRNA after 30 min incubation of the polyplexes (NP 15) at 30°C with RNase A. Significant differences in protection against RNase digestion compared to both polymers with the lowest degree of amine substitution: DEAPA-PVA(19) and DEAPA-PVA (23) are marked with ***$p < 0.001$. (**C**) Correlation between degree of amine substitution and % of siRNA remaining was calculated for 8 mIU/0.5 μg siRNA.

facilitating cytosolic release. Several positively charged polymers, such as PAMAM, PEI, diethylaminoethyl-dextran, were found capable of disrupting cellular compartments, inducing the formation of holes and thinning the membrane by electrostatic interactions (22,23). To study the membrane interaction between the DEAPA-PVA polyplexes and negatively charged vesicles, DPPC/DPPG liposomes in the molar ratio 9:1 were prepared and incubated with the polyplexes for 4 h. Carboxyfluorescein (CF) was encapsulated into these liposomes and served as a marker for membrane leakage. Treatment with Triton led to total release of CF from the liposomes and was set as 100%, PBS served as a blank. With a CF release of ~40%, the DEAPA-PVA polymers showed comparable membrane interaction to PEI 25 kDa (Fig. 9). DEAPA-PVA(48), the polymer with the highest amine density, displayed an even higher CF release than PEI 25 kDa. Incubation with Lipofectamine caused 70% release of CF, which is probably due to membrane fusion, followed by membrane destabilization and disruption. Although this is a simplified membrane model based on CF encapsulated in liposomes, it provides a good estimation of possible interactions between positively charged polyplexes with lipid model membranes. Furthermore, the results were in good agreement with the observations of Leroueil *et al.*, who showed by atomic force microscopy how cationic nanoparticles are capable of disrupting lipid bilayers (22).

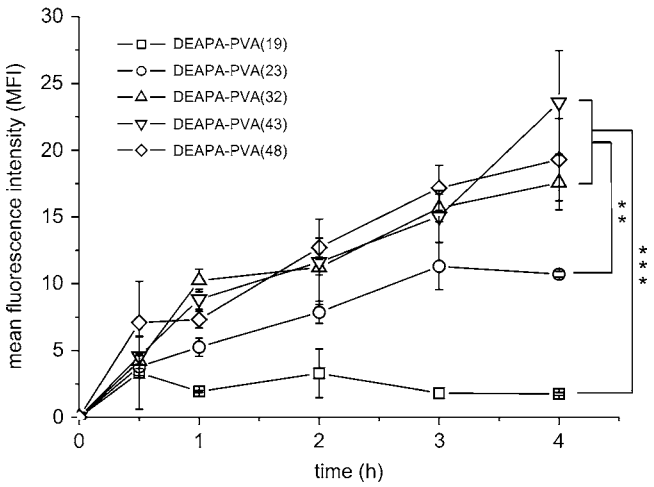

Fig. 7 Cellular uptake of DEAPA-PVA/siRNA polyplexes into SKOV-luc cells studied by flow cytometry at different incubation times. Significant differences in uptake after 4 h are marked with asterisks *** $p < 0.001$, ** $p < 0.01$.

Knockdown Efficiency

The gene silencing efficacy of the DEAPA-PVA polymer series at different NP ratios was investigated in SKOV-luc

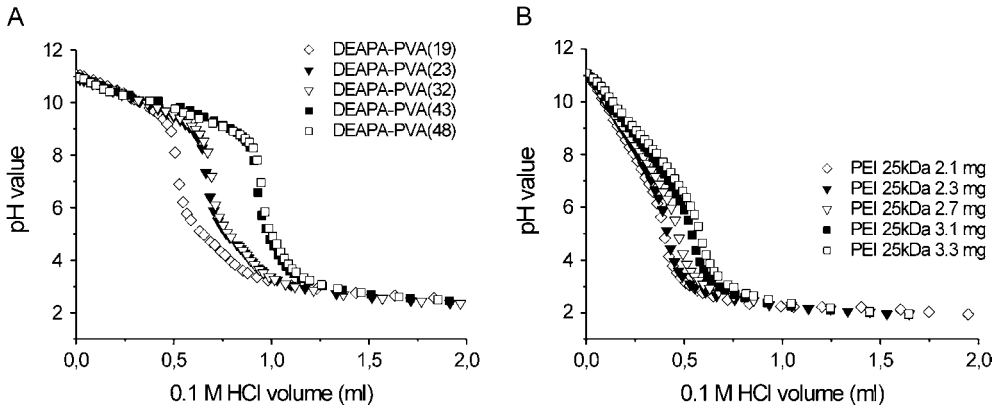

Fig. 8 Buffer capacity of DEAPA-PVA polymers (**A**) compared to PEI 25 kDa at the same amine mol concentration (**B**) studied by base-acid titration with 0.1 M HCl from pH 11 to pH 2.

cells stably expressing firefly luciferase. As displayed in Fig. 10, negligible knockdown of luciferase activity was observed for DEAPA-PVA(19) and DEAPA-PVA(23) polyplexes, both showing insufficient siRNA protection and intracellular uptake. DEAPA-PVA(32) displayed a significant reduction to 35% in gene expression at NP ratio 20 compared to untreated cells. While the polymers with the highest amine density, DEAPA-PVA(43) and DEAPA-PVA (48), also reduced luciferase activity to 20–30% of untreated control cells, non-specific knockdown (~20–50%) was also increased. PEI 25 kDa did not show any significant gene silencing effects at NP 7 and 10. At NP 15 and 20, knockdown was accompanied by unspecific off-target effects of PEI 25 kDa. Too high binding affinity to siRNA, as demonstrated in the ethidium bromide exclusion assay, might have led to insufficient release of the siRNA in the cytosol. This is most likely the main factor for inefficient gene silencing. Lipofectamine was used as a standard in this experiment and served as a comparison.

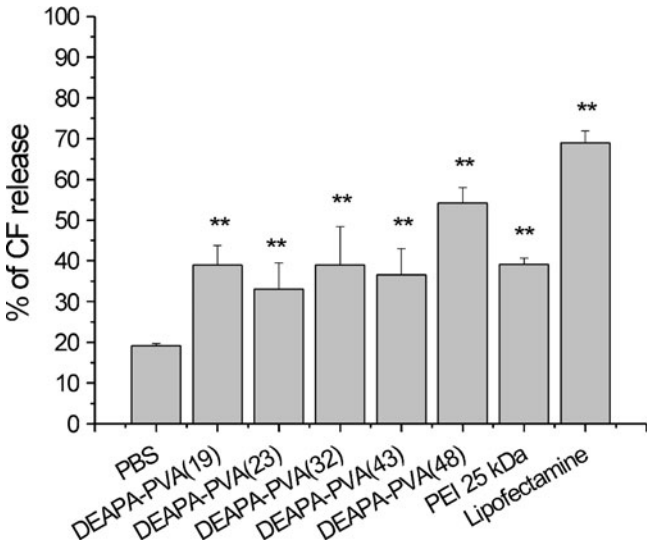

Fig. 9 Membrane interactions of DEAPA-PVA/siRNA polyplexes, PEI/siRNA polyplexes and Lipofectamine 2000/siRNA using CF encapsulating liposomes as a membrane model. Significant differences compared to the negative control PBS were marked with $^{**}p < 0.01$. Triton-X 100 served as positive control (= 100%).

DISCUSSION

There are many factors influencing effective polymer-mediated siRNA delivery, such as cellular uptake, siRNA protection against degrading enzymes and endosomal release into the cytosol (3). Polymers, such as PEI, which proved to be effective pDNA delivery vectors were shown to be less effective in gene silencing, indicating that different structural properties of polymers are required for gene and RNAi delivery (24,25). These different structural requirements are most likely based on the large difference in size—siRNA only consist of 21–23 bp of nucleic acids, while pDNA can be up to several kilo base pairs long (26)—and the different site of actions. siRNA only needs to be delivered into the cytosol, whereas the intracellular transport of pDNA into the nucleus is a crucial step. To contribute to the understanding of a polymer structure and its performance in siRNA delivery, we investigated the structure-activity relationship of a series of tertiary amine-modified poly vinyl alcohols (PVA) and the impact of their amine density on gene silencing. By varying the degree of amine substitution, from 19% to 48% of the PVA backbone, the impact of the spacing of the tertiary amine blocks on the siRNA binding affinity, protection of the RNA against degrading enzymes and gene silencing was studied. It was found that a certain degree of amine substitution was necessary to mediate efficient gene silencing, whereas a further increase in amine density led to unspecific off-target effects.

This class of polymers has been applied for gene delivery in our research group before. Wittmar *et al.* investigated the pDNA polyplex formation and transfection efficiency dependency on the degree of amine substitution. The limiting factor for the low transfection efficiency of DEAPA-PVA polymers with pDNA was the low amine density (12). Enhancing transfection efficiency could be approached by either grafting PLGA chains onto the

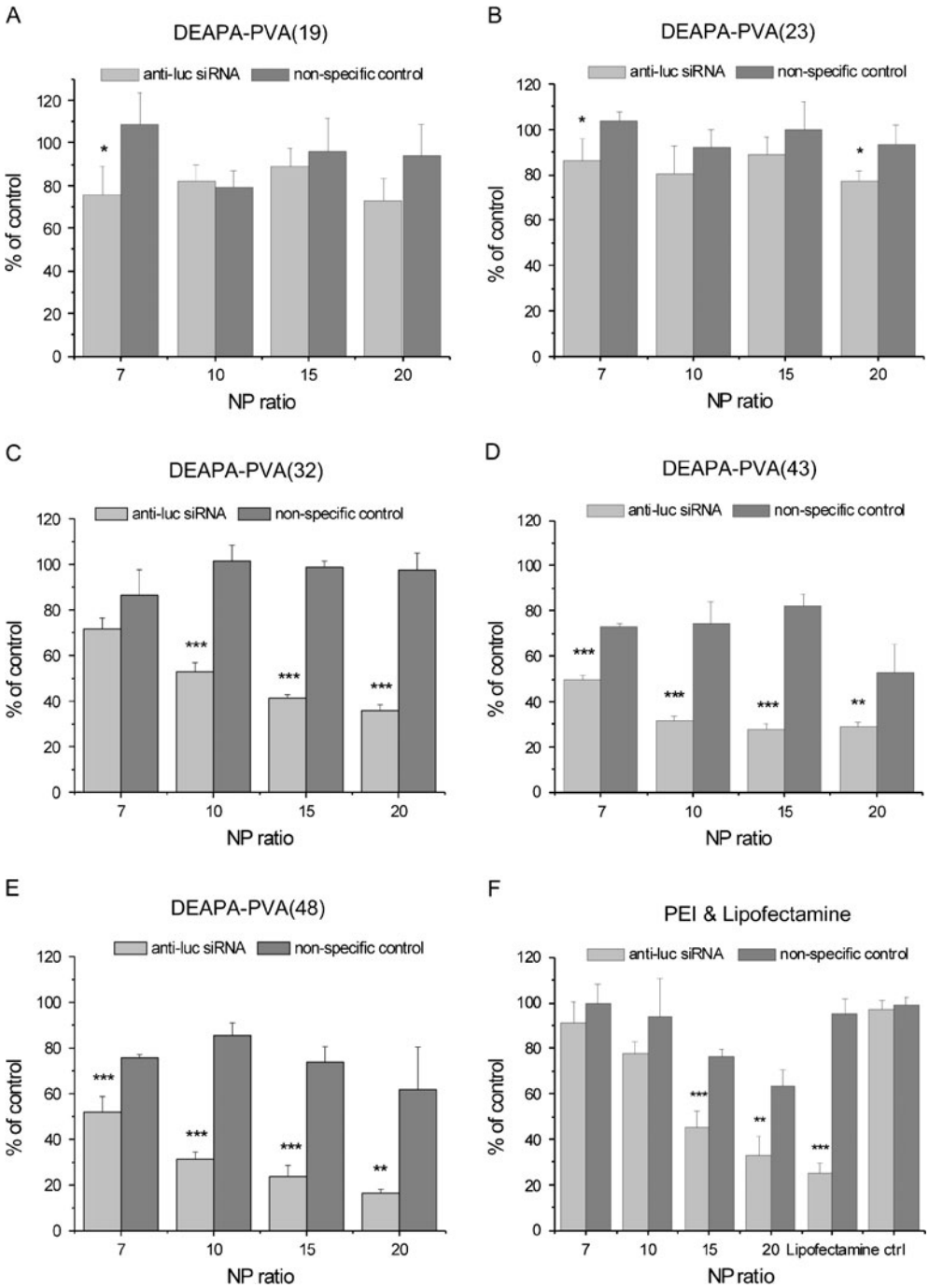

Fig. 10 Luciferase knockdown of polyplexes in SKOV-luc cells at different NP ratios **A**) DEAPA-PVA (19), **B**) DEAPA-PVA (23), **C**) DEAPA-PVA(32), **D**) DEAPA-PVA (43), **E**) DEAPA-PVA(48), **F**) PEI 25 kDa and Lipofectamine/siRNA. Significant differences compared to non-specific siRNA were marked with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

DEAPA-PVA backbone (27,28) or by increasing the amine density. Grafting of PLGA chains to the DEAPA-PVA backbone leads to water-insoluble polymers with the ability to encapsulate siRNA or DNA into rapidly biodegradable nanoparticles with high transfection and knockdown efficiency. Upon degradation of the PLGA chains, which is accelerated through the presence of amines (29), siRNA is released from these nanoparticles in a controlled manner (27). The objective of this study was to optimize the water-soluble PVA-DEAPA polymers for siRNA delivery by adjusting the amine density. A focussed library of DEAPA-PVA polymers with increasing degree of amine substitution (19% and 23% to 32%, 43% and 48%) was synthesized. As shown in the knockdown data, no gene silencing could be achieved when using the two DEAPA-PVA polymers with the lowest amine density of 19% and 23%. These amine densities of DEAPA-PVA are comparable to the highest one used by Wittmar *et al.* (12), where very low transfection efficiency with pDNA was achieved. However, significant knockdown was possible by further increasing the amine density to 32–48%: DEAPA-PVA

(32), DEAPA-PVA(43) and DEAPA-PVA(48). We found a strong correlation between amine density, siRNA complexation efficiency, protection against degrading enzymes and intracellular uptake, which are all critical factors for effective gene silencing (30). The two polymers with the lowest degree of amine substitution, DEAPA-PVA(19) and DEAPA-PVA (23), not only showed very weak siRNA binding affinity and compaction but also mediated very little siRNA uptake into SKOV-luc cells. DEAPA-PVA(19) ranged slightly above background level. Low intracellular uptake and weak siRNA complexation combined with insufficient protection against RNase are most likely the main factors for the lack of gene silencing. Although free or unbound siRNA could only be slightly reduced from 64% to 60% when forming DEAPA-PVA(32)/siRNA polyplexes, this polymer showed higher intracellular uptake after 4 h and significantly higher protection against RNase than the DEAPA-PVA(19) and DEAPA-PVA(23) polymers. Knockdown efficiency of 65% compared to cells treated with a non-specific siRNA sequence was obtained when incubating the cells with DEAPA-PVA (32) polyplexes at NP 20. Specific reduction of luciferase activity was also observed with the higher amine-substituted polymers DEAPA-PVA(43) and DEAPA-PVA(48), albeit with a 40–50% increase in non-specific knockdown at NP 20. This is most likely caused by carrier toxicity as shown in the MTT-assay. Previous studies have also shown that polyplexes at higher NP ratios can induce toxicity due to the presence of free polymers (31) and mediate unspecific knockdown effects (8). While PEI 25 kDa promoted a four-fold higher siRNA uptake than the DEAPA-PVA polymers, it did not show higher knockdown efficiency. This implied that a compromise between sufficient complexation and not too high binding affinity is important (19,24). As the same NP ratios have been applied for all polymers, the observed increase in siRNA complexation with increasing amine density of the polymers is most likely attributed to the spacing between the tertiary amines in the modified PVA backbone. The smaller spacing between the tertiary amine groups in the DEAPA-PVA polymers with higher degree of amine substitution did not only facilitate better siRNA complexation but also led to improved siRNA protection, intracellular uptake and gene silencing, which is in good agreement with data obtained by Lin *et al.* and Liu *et al.* (32,33). The reason why these DEAPA-PVA polymers promote gene silencing despite their lack of buffering capacity in the pH range of 7.4–5.0 has yet to be investigated. One possible explanation may be attributed to the catalytic effect of siRNA based on the fact that only a few siRNA molecules per cell were necessary to achieve complete gene silencing as shown by Fire *et al.* (34). Furthermore, recent studies have demonstrated that apart from buffering capacities, other factors, such as membrane interaction properties of the polymer, may play an important role in endosomal escape. Leroueil *et al.* suggested that a variety of nanoparticles prepared from polyamidoamine dendrimers (PAMAM), PEI and diethylaminoethyl-dextran can disrupt lipid bilayers and induce the formation of holes in such vesicles (22). Reineke and coworkers and Funhoff *et al.* showed that buffering capacity cannot solely be used to predict the transfection ability of a polymer but that other factors, such as intracellular uptake, complexation efficiency and protection of the nucleic acid against degrading enzymes, are also important (33,35). For future *in vivo* applications, the DEAPA-PVA polymers require further optimization with regard to siRNA complexation, polyplex stability and polyplex size. This is especially important, as it has been shown by several groups that even highly stable polyplexes *in vitro* can dissociate rapidly under *in vivo* conditions (36,37).

CONCLUSION

In conclusion, we could show that siRNA complexation efficiency, RNA protection against degrading enzymes and gene silencing could be controlled by the number of tertiary amines in the modified PVA backbone. A strong correlation between the degree of amine substitution and siRNA complexation as well as protection of RNA against RNase was observed. From the series of DEAPA-PVA polymers tested, DEAPA-PVA(32) showed the most efficient protein knockdown. Future studies will focus on the optimization of smaller polyplex sizes, polyplex stability, and fewer off-target effects. This class of polymers was shown to mediate efficient gene silencing and offers a promising platform for further structural modifications.

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