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Biodegradable, Cationic Methacrylamide-Based Polymers for Gene Delivery to Ovarian Cancer Cells in Mice

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Abstract: A series of cationic, methacrylamide polymers was tested for use as a biodegradable gene carrier in ovarian cancer. Tumor transfection activity of polyplexes consisting of a reporter gene and different methacrylamide polymers was assessed, after intraperitoneal injection in mice bearing an ovarian cancer xenograft. In this model, polyplexes based on poly(HPMA−DMAE) showed transfection activity similar to polyplexes based on the nondegradable and rather toxic polyethylenimine (PEI22). The tumor transfection activity of the pHPMA−DMAE polyplexes was remarkable considering their poor transfection activity in in vitro assays. Polyplexes based on pHPMA−DMAE were devoid of any cytotoxicity and mediated highest transfection activity at the highest N/P ratio investigated. Tumor cell gene expression after a single administration of these polyplexes rapidly declined within time, at a similar rate to that observed after injection with polyplexes based on PEI22. Incubation of the polyplexes with hyaluronic acid (HA), a polyanion accumulating in the ascitic fluid of ovarian cancer bearing mice, changed the physical characteristics of the pHPMA−DMAE and PEI22 polyplexes. The transfection activity of PEI22-based polyplexes, but not that of pHPMA−DMAE based polyplexes, was strongly impaired by HA. Differences in HA sensitivity might have contributed to the in vivo gene expression activities of pHPMA−DMAE- and PEI22-based polyplexes. pHPMA−DMAE-based polyplexes have potential for use in ovarian cancer therapy due to their considerable transfection activity, their low cytotoxicity, and their HA resistance.

Keywords: Methacrylamide polymers; ovarian cancer; biodegradable; cytotoxicity; hyaluronic acid; gene delivery; gene therapy

Introduction

Ovarian cancer is a frequently occurring malignancy disorder with a high mortality rate. Detection occurs at advanced cancer stages when the ovarian capsule has disrupted and clusters of exfoliated tumor cells spread across the peritoneal cavity. Intraperitoneal dissemination is commonly associated with the formation of ascites, the accumulation of fluid within the peritoneum.[1,2] Resistance against current chemotherapeutics often develops rapidly.[1] Prognosis might be improved by new innovative strategies such as anticancer gene therapy, applicable in the advanced stage of ovarian cancer.[3,4]

Ovarian cancer cells tend to remain localized in the peritoneal cavity, allowing for the regional delivery of the gene of interest.[3] Cationic polymers have been shown to greatly improve gene transfer to the peritoneal tumor cells

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in different ovarian cancer models. Louis et al. demonstrated significant reporter gene expression in the tumor nodes present in the peritoneal cavity of ovarian cancer-bearing mice, after i.p. administration of polyplexes based on the linear polymer polyethyleneimine (22 kDa, PEI22).[5] Interestingly, the i.p. delivery of DNA delivery systems enables a relative tumor cell selectivity. This is not only due to the particular peritoneal organization, which allows passive targeting of the polyplexes, but also due to the susceptibility of the proliferating tumor cells to nonviral gene delivery.[5] Nevertheless, for efficient transfection of the ovarian cancer cells in the peritoneal cavity, high and frequent dosing of polyplex appears to be needed.[5,6] This is likely the result of the inhibiting effects of hyaluronic acid and other polyanions, accumulating in the ascitic fluid.[5,7–10] The toxicity of the cationic polymers tested for gene transfer in ovarian cancer so far—poly(2-(dimethylamino)ethyl methacrylate) and PEI22—poses a potential drawback when frequent and high dosage regimes are warranted.[5,6,11]

Recently, a series of biodegradable, cationic methacrylamide-based polymers has been studied for in vitro gene delivery to cancer cells.[12] These polycationic polymers are biodegradable in a sense that the carbonate esters connecting the cationic side group and the polymer backbone are hydrolyzed, yielding pHPMA as degradation product. Although the polymers have an average molecular weight range that exceeds the maximal molecular weight for glomerular filtration (45 kDa), introduction of biodegradable linkers in the pHPMA backbone is a feasible strategy to allow efficient renal clearance of the then-formed low molecular weight pHPMA fragments. As a result of the degradation, polyplexes based on these polymers destabilize within hours to days at physiological conditions and demonstrate a low cytotoxicity profile. We reasoned that these methacrylamide-based polymers would be suitable carriers for the i.p. delivery of DNA to ovarian cancer tumor cells. Their low cytotoxicity might allow the administration of polyplexes at polymer concentrations high enough to counteract the above-mentioned negative effects of polyanions. In this study, we investigated the transfection activity of polyplexes based on cationic methacrylamide polymers in the OVCAR-3 ovarian cancer mouse model. As a reference, we used polyplexes based on the nondegradable polymer PEI22, a cationic polymer referred to as the gold standard in polymer-mediated gene delivery.[5]

Materials And Methods

Materials. Plasmid encoding for firefly luciferase, pcDNA3Luc, was produced by Plasmid Factory (Bielefeld, Germany). The plasmid is under the transcriptional control of the cytomegalovirus immediate promoter. Luciferase assay reagent, reporter gene lysis buffer, and quantilum recombinant luciferase were obtained from Promega (Leiden, The Netherlands). Hyaluronic acid (HA, M_w ~2MDa) and ethidium bromide were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). *N*-(3-Aminopropyl)methacrylamide hydrochloride (NAPMAm) was obtained from Polysciences (Eppelheim, Germany). Linear polyethylenimine (PEI22, M_w 22 kDa) was a generous gift from Prof. Dr. E. Wagner (Ludwig-Maximilians-Universität München).[13]

The cationic methacrylamide-based polymers (pHPMA—DMAE, pHPMA—DEAE, pHPMA—DMAPr, pHPMA—MPPM, and pHPMA—DBMPAP, structures given in Figure 1) were synthesized as previously described.[12] INF-conjugated pHPMA—DMAE was synthesized by copolymerization of HPMA—DMAE (95 mol %) with NAPMAm (5 mol %), followed by conjugation of the reduced di-INF-7 peptide.[14] The INF conjugation efficiency was 38%, as determined by the measurement of the amount of released pyridine-2-thione (UV absorption).

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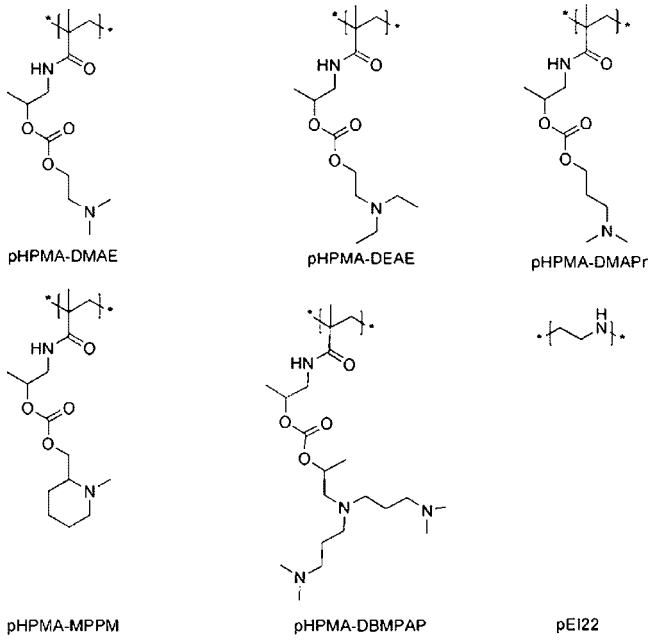

Figure 1. Chemical structures of the cationic methacrylamide polymers and linear PEI22.

Methods. Preparation of the Polyplexes. Polyplexes were prepared at different molar ratios of ionizable nitrogen to phosphate DNA (N/P ratio), ranging from 6 to 100. The mass per ionizable nitrogen of the methacrylamide units depended on the side group (pHPMA−DMAE 250, pHPMA−DEAE 286, pHPMA−DMAPr 272, pHPMA−MPPM 298, and pHPMA−DBMPAP 414 Da). The mass per nitrogen of PEI22 and the mass per phosphate of DNA were 43 and 330 Da, respectively. Polyplexes were prepared in 20 mM Hepes, 5% g/v glucose, pH 7.1 (HBG) at a DNA concentration of 150 μg/ml. In short, different amounts of polymer (depending on the N/P ratio) were dissolved in 450 μl of a 20 mM Hepes solution, pH 7.1. The polymer solution was added to 450 μl of 20 mM Hepes, pH 7.1, containing 150 μg DNA. The resulting dispersions were vortexed and incubated for 30 min at room temperature. Then, 100 μl of a 50% g/v D-glucose solution was added to obtain an isotonic dispersion.[13] For the in vivo experiments, polyplex dispersions were used as such. For the in vitro experiments, polyplex dispersions were diluted 15 times to a final DNA concentration of 10 μg/ml with either HBG, HBS (20 mM Hepes, 0.9% g/v NaCl, pH 7.4) or HBS to which 0.5 mg/ml HA was added.

Characterization of the Polyplexes. The average hydrodynamic diameter and the zeta-potential of the polyplexes were determined using dynamic light scattering (ALV CGS-3 system, Malvern Instruments, UK) and electrophoretic mobility measurements (Zetasizer Nano-Z, Malvern instruments, UK), 1 h after dilution of the polyplex dispersions in the different buffers (final DNA concentration 10 μg/ml). The instruments were calibrated using polystyrene latex beads of defined size and zeta-potential.

Binding of the different polymers to DNA was studied using an ethidium bromide (EtBr) fluorescence quenching assay.[15] Polyplexes were prepared in HBG, as described above with EtBr added to the DNA solution 10 min prior to the addition of the different polymers (molar ratio EtBr to DNA phosphates of 1:8). After incubation of the polyplex dispersions for 1 h and after subsequent dilution in the different buffers (HBS, HBS+HA), the fluorescence was quantified using a Fluostar Optima plate-reader (BMG Laboratory Tech, Germany). Fluorescence values were measured at an excitation/emission wavelength of 500 and 600 nm, respectively, and converted into relative, residual fluorescence (Fr) using the following equation:

$$F_r = (F_{obs} - F_e)/(F_{100} - F_e)$$

where F_{obs} is the observed fluorescence of the polyplex samples (DNA + polymer), F_e is the fluorescence of EtBr in the absence of DNA and polymer, and F_{100} is the fluorescence of EtBr in the presence of DNA and in the absence of polymer.

In Vitro Transfection. NIH:OVCAR-3 cells (human ovarian adenocarcinoma, ATCC HTB-161) were cultured in DMEM, 4.5 g/L of glucose completed with bovine calf serum (10%), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B at 37 °C and a 5% CO_2 humidified atmosphere.

Transfection and cytotoxicity studies were carried out as described previously.[16] In brief, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. After 24 h, the cells were overlaid with fresh medium (100 μL) and subsequently with a diluted polyplex dispersion (100 μL, 10 μg/mL of DNA, HBS, or HBS/HA). After incubation of the cells with the polyplexes for 1 h, the medium was refreshed and the cells were cultured for another 24 or 72 h. Cells were tested for reporter gene expression (transfection activity) and cell viability (cytotoxicity).

Transfection activity of the polyplexes was determined by measuring the luciferase expression by the transfected cells as follows. Cells were washed with phosphate-buffered saline (PBS) and subsequently lysed by the addition of 100 μL of reporter gene lysis buffer, followed by a single freeze/thaw cycle. Relative light units (RLU) of the samples were measured for 10 s at room temperature using a Berthold 9507 luminometer (EG&G Benelux BV, The Netherlands) after mixing of 20 μL of the cell lysate with 100 μL of the luciferase assay reagent. Twenty microliters of the remaining lysates was used for the determination of the protein concentration, using a Micro BCA protein assay kit (Pierce, The Netherlands). Gene expression was calculated as picograms of luciferase per milligram of protein, using the recombinant luciferase standard.

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Cytotoxicity of the polyplexes was determined using an XTT colorimetric assay, based on the metabolic, cellular reduction of a tetrazolium reagent.[17] Cell viability was expressed as percentage of viable cells 24 or 72 h after transfection, normalized against buffer-treated cells.

In Vivo Gene Expression Studies. The animal experiments were performed in line with national regulations and approved by the local animal experiments ethical committee. The NIH:OVCAR-3 cell line was propagated intraperitoneally in six to nine week old Balb/c athymic mice (Harlan, The Netherlands). After peritoneal inoculation, a mouse model for ovarian cancer develops, which is characterized by the formation of ascites and clusters of fast proliferating tumor cells which are confined to the peritoneal cavity.[7,18] At 3−4 week intervals, tumor cells were recovered from the donor animals by rinsing the peritoneal cavity twice with 5 mL of cold PBS.[8] For transfection studies, mice were inoculated by i.p. injection of 1×10^7 OVCAR-3 cells in PBS, obtained from the peritoneal lavage of the donor mice. Five days after inoculation, the mice were injected i.p. with the different polyplex dispersions (0.2 mL, 150 μg/mL of DNA). At different time points after administration, the mice were sacrificed by CO_2 asphyxiation. Tumor cells were recovered by rinsing the peritoneal cavity twice with 5 mL of PBS. The total number of tumor cells was determined by counting the cell nuclei density of a fraction of the peritoneal lavage. In short, cells were lysed by osmotic shock (Zap-Oglobin II, BeckmanCoulter) and counted using a cell counting chamber. In the general experimental setup (peritoneal lavage at 24 h after transfection), 6–10 $\times$ 10^7 cells were recovered per mouse. In vivo transfection activity of the polyplexes was determined by measuring luciferase expression by the recovered tumor cells and the major organs. The peritoneal lavage was centrifuged and the tumor cell pellets and organs were homogenized in 0.5−1 mL of reporter gene lysis buffer, using a tissue homogenizer. The tissue homogenates were incubated on ice for 30 min, vortexed, and subsequently centrifuged at 12000*g* for 10 min. Relative light units (RLU) of the samples were measured for 10 s at room temperature using a Berthold 9507 luminometer after mixing of 20 μL of the supernatant with 100 μL of the luciferase assay reagent. The transfection activity of the polyplexes was expressed as RLU per organ or RLU per 1×10^8 tumor cells.

Results and Discussion

Methacrylamide-Based Polyplexes: In Vivo Transfection Activity. Polyplexes based on the different cationic methacrylamide polymers (Figure 1) were tested for their tumor transfection efficiency in the OVCAR-3 ovarian cancer mouse model.

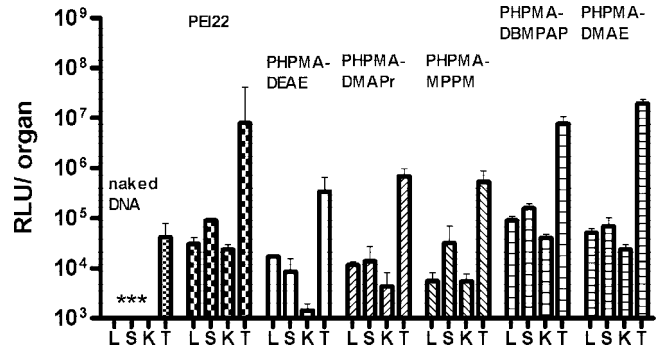

Figure 2. Tumor and organ luciferase expression, 24 h after i.p. injection of the different methacrylamide-based polyplexes (N/P 50), uncomplexed naked DNA, or PEI22 based polyplexes (N/P 6). L: liver, S: spleen, K: kidney, T: tumor/1 $\times$ 10E8 tumor cells. Thirty micrograms of DNA was injected in all cases (mean + deviation of the mean, $n = 2$). *: below detection limit.

Polyplexes were prepared at an N/P ratio of 50 and administered intraperitoneally (i.p.), 5 days after inoculation of Balb/c athymic mice with 1×10^7 OVCAR-3 cells. A preparation consisting of naked DNA and a preparation consisting of polyplexes based on PEI22 (N/P 6) were used as reference formulations. The mean particle size of the different polyplexes, dispersed in 20 mM Hepes, 5% g/v of glucose, pH 7.1 (HBG), varied from 120 to 170 nm as measured by dynamic light scattering. The different formulations did not affect the amount of recovered tumor cells nor did they provoke any apparent, acute toxicity.

Tumor cell luciferase expression was 10- to 500-fold higher for the groups that received polyplexes based on the different methacrylamide polymers, as compared to the group that received the naked DNA formulation (Figure 2). Polyplexes based on pHPMA−DMAE yielded the highest tumor cell gene expression. The amount of luciferase protein, recovered per peritoneal lavage, was comparable to the amount obtained using PEI22-based polyplexes (2.7–3.8 ng and 0.8–1.9 ng for pHPMA−DMAE and PEI22, respectively). In vivo gene expression was relatively selective to the ascitic tumor cells, compared to the major organs, in good agreement with other publications.[5,19] Moderate gene expression was observed in the organs lined by the mesothelial layer (spleen, and to a lesser extent liver and kidney). The expression associated with these organs could very well be due to transfection of the mesothelial layer lining the peritoneal cavity and covering these tissues.[6] Low or undetectable gene expression was observed in the heart and the lungs of the mouse.

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The observed in vivo tumor cell transfection activity of the different methacrylamide-based polyplexes did not correlate with the activities found in an earlier in vitro study.[12] In the in vitro study, using COS-7 cells (African monkey kidney fibroblast-like), pHPMA−DMAE-based polyplexes did not show any transfection activity, whereas pHPMA−DEAE- and pHPMA−MPPM-based polyplexes were most active. Poor in vitro−in vivo correlations have been reported earlier in systemic and intratumoral gene delivery and are likely to be a consequence of the polymer or polyplex interaction with nontarget agents in vivo.[20]

Because of their encouraging in vivo transfection activity, polyplexes based on the polymer pHPMA−DMAE were selected for a more in-depth investigation of their transfection properties.

pHPMA−DMAE-Based Polyplexes: Degradation and Cytotoxicity. Previously, it was shown that due to the biodegradable character of the different pHPMA derivatives, polyplexes based on these polymers destabilized at physiological conditions (pH 7.4, 37 °C), enabling the controlled release of the DNA.[12,21] Side-chain hydrolysis reduces the polymer charge density and thereby impairs the DNA condensing and binding capacity, ultimately leading to polyplex destabilization and dissociation. Similar to the destabilization rate found for preparations at a low DNA concentration (10 μg/mL of DNA),[12,21] pHPMA−DMAE polyplexes prepared for in vivo use (150 μg/mL of DNA) destabilized within 1 day after incubation at physiological conditions. In contrast, polyplexes based on PEI22 remained stable for at least 7 days of incubation (data not shown).

Previously, the different methacrylamide-based polyplexes showed very moderate cytotoxicity toward COS-7 cells.[12,21] Figure 3 shows the difference in cytotoxicity of pHPMA−DMAE and PEI22 polyplexes toward OVCAR-3 cells, the ovarian cancer cell line used throughout this study. Twenty-four hours after incubation with the PEI22 polyplexes prepared at an N/P ratio of 25 (corresponding to a polymer concentration of 32 μg/mL), the relative cell viability was reduced to 50% (Figure 3B). Seventy-two hours after incubation, cell viability had further dropped to 14%. Incubation of the cells in the absence of serum reduced cell viability with an additional 10–15% under the conditions evaluated. The presence of negatively charged serum proteins can partially mask the cytotoxic effects of PEI22 by binding to free polymer or by preventing salt-induced aggregate formation.[22] Interestingly, cells incubated with pHPMA−DMAE polyplexes prepared at N/P ratios up to 100 (corresponding with a polymer concentration of 800 μg/mL) did not show any reduction in cell viability under the conditions evaluated (24 or 72 h after transfection, in the presence or in the absence of serum). Cytotoxicity of cationic polymers has been attributed to damaging effects on membrane integrity, as a result of the multiple attachment of these polymers to the negatively charged outer and inner cell membranes.[23] The observed lack of cytotoxicity of the pHPMA−DMAE-based polyplexes might be partly attributed to the biodegradable character of the polymer, as this leads to the removal of the polymeric cationic charge, and thereby prevents interaction of pHPMA−DMAE with cellular membranes.

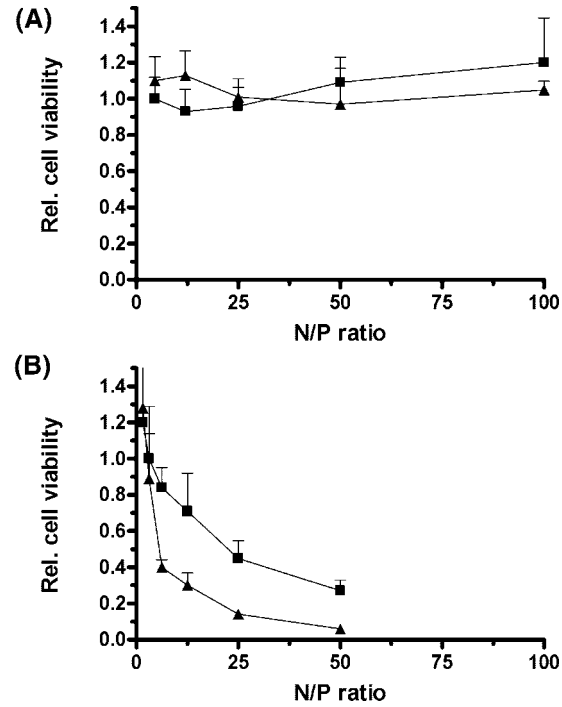

Figure 3. Relative cell viability of OVCAR-3 cells, 24 (■) and 72 h (▲) after incubation with pHPMA−DMAE- (A) and PEI22-based polyplexes (B). Values are normalized against HBS-treated OVCAR-3 cells (mean + SD, $n = 3$).

pHPMA−DMAE-Based Polyplexes: In Vivo Transfection Activity. The polymer-to-DNA dependency of the tumor cell gene expression by pHPMA−DMAE and PEI22 polyplexes was investigated. Figure 4A shows that the tumor cell transfection activity of pHPMA−DMAE-based polyplexes increased with the N/P ratio investigated (e.g., transfection activity at N/P 50 was 500-fold higher than that observed at N/P 6). Similar to an earlier study with low molecular weight PEIs, the transfection activity of the polyplexes is enhanced by the presence of additional free polymer, without having

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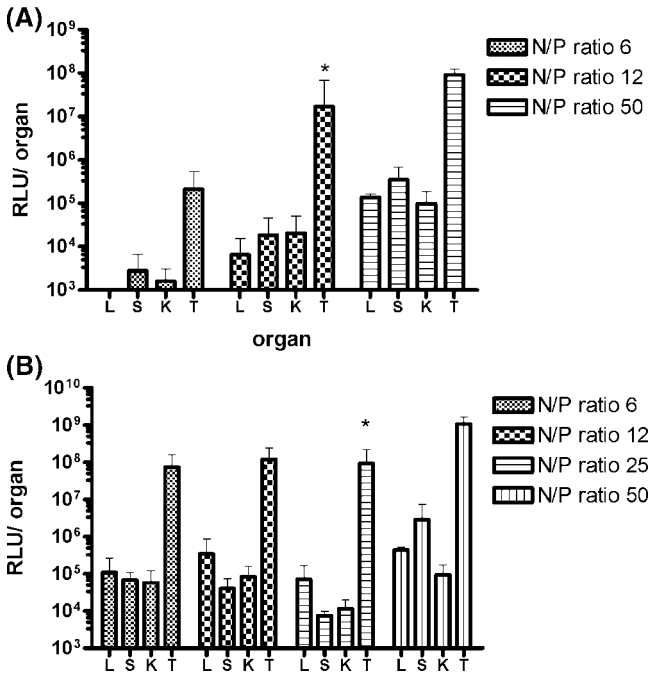

Figure 4. Tumor and organ luciferase expression, 24 h after i.p. injection of pHPMA−DMAE (A) and PEI22-based polyplexes (B), prepared at different N/P ratios: L, liver; S, spleen; K, kidney; T, tumor/1 × 10E8 tumor cells. Thirty micrograms of DNA was injected in all cases (mean + SD, $n = 3$, *: $n = 2$).

any effect on the cell viability.[24] The mechanism by which free polymer adds to the transfection activity of gene delivery complexes could be by its binding to anionic macromolecules in the extracellular space, thus protecting the complexes from destabilization. Alternatively, the free polymer might bind to cells, thereby altering the cell binding and uptake pathways of the gene delivery complexes. Luciferase expression in the main organs lined by the peritoneal mesothelial layer concomitantly increased with 2 orders of magnitude. For the PEI22 polyplexes, tumor gene expression was not affected when the polymer-to-DNA ratio increased from 6 to 25 (Figure 4B). Presumably, PEI22 polyplexes mediate efficient transfection activity which is less dependent on additional free polymer. Administration of PEI22 polyplexes at the highest N/P ratio investigated (N/P 50) enhanced tumor gene expression 10-fold compared to the lower N/P ratios. However, mice receiving the latter formulation experienced apparent side-effects within the first hours after administration (increased spinal curvature and isolation behavior). No toxicity was observed in any of the other groups. The different formulations did not affect the amount of recovered tumor cells in any of the experimental groups.

The kinetics of transgene expression were studied, after a single i.p. administration of pHPMA−DMAE or PEI22

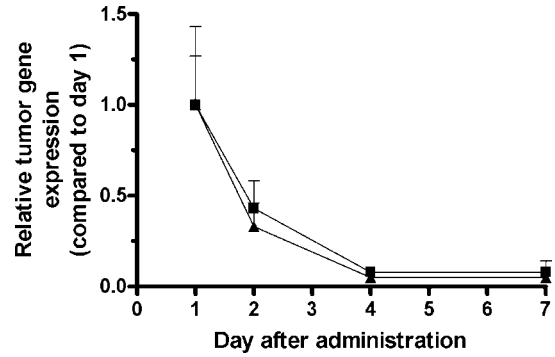

Figure 5. Tumor luciferase expression, 1−7 days after a single i.p. injection of pHPMA−DMAE- (■) and PEI22-based (▲) polyplexes. Thirty micrograms of DNA was injected (N/P 50 and 6, respectively). Gene expression values are normalized to the gene expression levels obtained 1 day after transfection (mean + SD, $n = 3$).

polyplexes (Figure 5). To study the transgene expression kinetics, the enzyme firefly luciferase is a suitable protein as it has a short half-life of 4 h, enabling the determination of "freshly produced" luciferase levels.[25] Tumor gene expression levels decreased rapidly over time, regardless of the type of carrier used. Four days postinjection, tumor gene expression had dropped to 8 and 5% of the levels at day 1, for pHPMA−DMAE and PEI22 polyplexes, respectively. Luciferase expression in the major organs was detectable only at day 1 and 2 (data not shown). The amount of recovered tumor cells steadily increased from 6×10^7 to 19×10^7 cells during the experiment as a result of tumor cell proliferation and was not affected by the different formulations. Previously, Louis et al. also found a short-lived gene expression of tumor cells after administration of PEI22 polyplexes.[5] The transient expression observed for PEI based polyplexes has been ascribed to their inherent toxicity toward transfected cells.[23,24,26] It was hypothesized that the lack of cytotoxicity of pHPMA−DMAE based polyplexes might prolong the tumor transgene expression as a higher number of surviving transfected cells is likely to prolong the production of the desired protein. However, the data from this study do not confirm this, which indicates that the contribution of polyplex induced cytotoxicity, in determining the apparent gene product elimination is overruled by other cellular events, like the loss of the episomal DNA upon cell division, DNA degradation or impairment of DNA transcription.[27,28]

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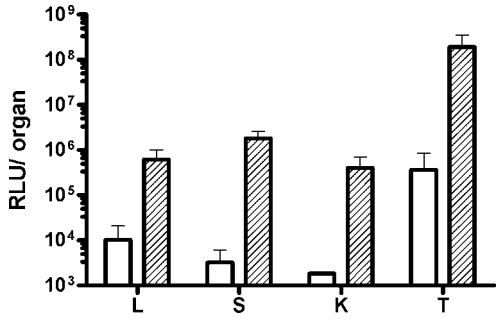

Figure 6. Tumor and organ luciferase expression, 24 h after i.p. injection of polyplexes based on INF conjugated pHPMA−DMAE copolymer (white bars) and unconjugated pHPMA−DMAE copolymer (hatched bars): L, liver; S, spleen; K, kidney; T, tumor/1 × 10E8 tumor cells. Thirty micrograms of DNA was injected (N/P 50, mean + SD, $n = 3$).

Table 1. Mean Size (nm), ζ Potential (mV), and DNA-Binding (Determined as Percent Residual DNA− EtBr Fluorescence, Res Fluor) of pHPMA−DMAE and PEI22 Polyplexes after 1 h of Incubation in HBG, HBS, or HBS Containing Hyaluronic Acid (HA) (mean (±SD); $n = 3$)

		pHPMA−DMAE		PEI22	
		N/P 6	N/P 50	N/P 6	N/P 50
HBG	size	170 (±10)	130 (±10)	150 (±20)	120 (±5)
	ζ-potential	+30 (±5)	+35 (±6)	+30 (±7)	+37 (±6)
HBS	size	700 (±160)	>1000	>1000	880 (±40)
	ζ-potential	±	+	±	+
	res fluor	38 (±2)	36 (±3)	20 (±2)	29 (±2)
HBS + HA	size	900 (±170)	960 (±40)	490 (±20)	570 (±20)
	ζ-potential	−	−	−	−
	res fluor	55 (±2)	56 (±2)	41 (±2)	43 (±2)

pHPMA−DMAE based polyplexes are considered to have limited endosomal escape properties.[12,14] It was shown that the in vitro transfection activity of pHPMA−DMAE-based polyplexes was strongly improved by the covalent attachment of the membrane destabilizing peptide INF-7 to the polymer. Therefore, the tumor transfection activity of polyplexes based on an INF-conjugated pHPMA−DMAE copolymer was also studied in vivo. The transfection activity of polyplexes based on the unconjugated polymer (Figure 6, hatched bars) was as similar as observed for polyplexes based on the homopolymer (Figure 2). In contrast, INF-conjugation of the copolymer strongly impaired gene expression by approximately 500-fold, in the tumor cells as well as in the major organs. The particle characteristics of the polyplexes based on the INF-conjugated copolymer were similar as polyplexes based on the homopolymer (mean size 150 nm, mean ζ potential +35 to +38 mV at N/P 50). The discrepancy between the in vitro and in vivo results might be a result of the interaction of the polyplexes with nontarget compounds, present in the peritoneal cavity of ovarian cancer bearing mice but absent in the in vitro experimental settings (see the following section).

pHPMA−DMAE-Based Polyplexes: Interaction with Hyaluronic Acid. The development of ascites in ovarian cancer is characterized by the peritoneal accumulation of polyanions such as hyaluronic acid (HA), either in dissolved form or associated to tumor and mesothelial cells.[9,10] Whereas serum proteins−which also accumulate in the ascitic fluid−have a limited effect on the in vitro transfection activity of PEI22[29,30] and pHPMA−DMAE based polyplexes (data not shown), these polyanions are known for their strong interference with positively charged gene delivery systems.[8,31,32] Therefore, the effect of HA on the biophysical properties and transfection activity of pHPMA−DMAE and PEI22 polyplexes was studied.

Mean size, ζ potential, and DNA binding strength of the polyplexes was studied, 1 h after incubation in HBG, HBS, or HBS containing 0.5 mg/mL of HA, a concentration relevant to ascitic fluid.[9,33] In contrast to polyplexes incubated in HBG, which were characterized by a small size and a highly positive ζ potential (120 to 170 nm, +30 to +37 mV, Table 1), the incubation of the polyplexes in HBS or HBS containing HA led to the formation of aggregates (size 500 to >1000 nm) and a reduction in ζ potential. The observed reversal of the ζ potential of the polyplexes, upon incubation with HA, points to binding of HA to the surface of the particles. Under the evaluated conditions, DLS analysis showed that HA did not yield detectable complexes with the free polycations. HA also caused a reduction of the DNA binding strength of both pHPMA−DMAE and PEI22, as was reflected by the recovery of the EtBr fluorescence signal (38 and 20% in the absence of HA, to 55 and 41% in the presence of HA, for pHPMA−DMAE- and PEI22-based polyplexes, respectively at N/P 6). Incubation of the polyplexes with HA did not lead to polyplex dissociation−and subsequent DNA release (residual fluorescence 100%). Therefore, we conclude that HA is not able to release DNA from the polyplexes which might then be subsequently degraded by nucleases.

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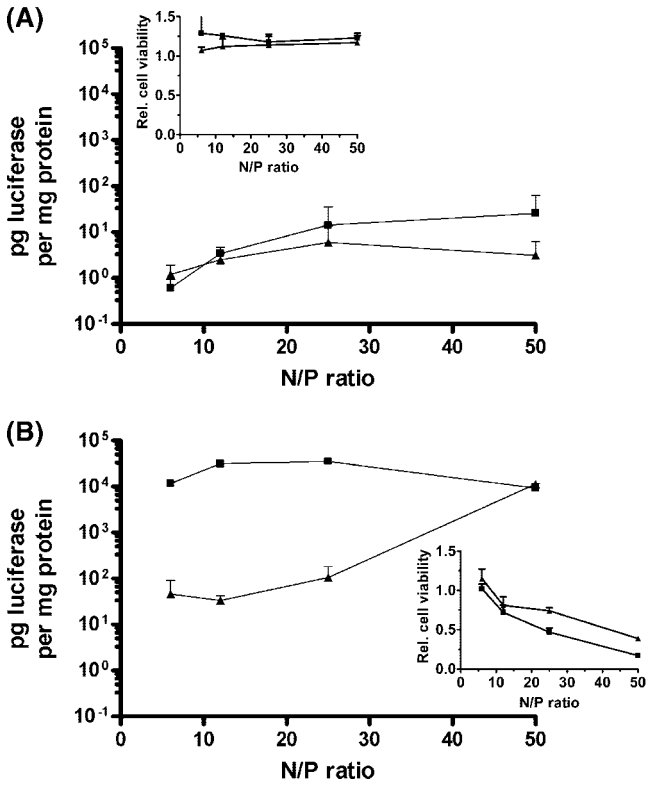

Figure 7. In vitro luciferase expression and relative cell viability (inset) of OVCAR-3 cells, 24 h after transfection with pHPMA_DMAE polyplexes (A) or PEI22-based polyplexes (B). Polyplexes were diluted in HBS (■) and HBS-containing hyaluronic acid (0.5 mg/mL) (▲) before addition to the cells. Cell viability values are normalized against HBS or HBS + HA treated OVCAR-3 cells (mean + SD, $n = 3$).

Figure 7 shows the effect of HA on the in vitro transfection activity of pHPMA−DMAE and PEI22 based polyplexes in OVCAR-3 cells. The inserts represent the cell viability data after incubation with the different formulations. In the absence of HA, incubation with pHPMA−DMAE based polyplexes gave low luciferase expression levels with no distinct optimal N/P ratio (Figure 7A). The lack of cytotoxicity of the pHPMA polyplexes enabled gene expression even at high N/P ratios when excess free polymer was present. The presence of HA did not affect gene expression mediated by pHPMA−DMAE polyplexes. Transfection with PEI22 polyplexes in the absence of HA resulted in a highly efficient gene transfer (Figure 7B), in line with earlier findings on ovarian cancer cells.[4] Highest luciferase levels (31−35 ng of luciferase per milligram protein) were observed at N/P ratios of 12−25. The presence of HA reduced the transfection activities of PEI22 polyplexes with 2−3 orders of magnitude. PEI22 polyplexes prepared at the highest N/P ratio (N/P 50) were resistant toward HA. Likely, the excess of free cationic polymer at this N/P ratio allows the PEI22 polyplexes to overcome the inhibitory effects of the polyanion. Alternatively, the observed increase in gene expression at this N/P ratio might be partly the result of the somewhat restored cell viability, observed in the presence of HA.

The in vitro transfection activity of PEI22 polyplexes was 3−4 orders of magnitude higher, as compared to pHPMA−DMAE polyplexes, irrespective of the applied N/P ratio. In contrast, when the in vivo transfection activity of the both polyplex systems was compared, the difference was much less pronounced (10-fold, at N/P ratios of 12 and higher). The presence of HA in the ascitic fluid of the OVCAR bearing-mice is likely to have added to this remarkable finding. We demonstrated that HA strongly impaired PEI22-mediated transfection activity, whereas the transfection activity of pHPMA−DMAE-based polyplexes was resistant against HA. Previously, the interaction of polyanions with the polyplexes has demonstrated to affect the transfection activity of different polyplex systems to a different extent. Polyplexes based on polymers with a significant endosomal escape capacity, like PEI, have been shown to be much more sensitive to the effects of HA than polyplexes based on polymers without endosomal buffering capacity (e.g., poly L-lysine based polyplexes).[31,34] The data presented in this study are in good agreement with these previous observations. Polyplexes based on PEI22 were found to be highly sensitive for HA, whereas polyplexes based on pHPMA−DMAE−a system which is regarded to have very limited endosomal escape properties−were found to be resistant toward interaction with HA. We speculate that the disappointing in vivo transfection activity of the INF-conjugated pHPMA−DMAE polyplexes might also be the consequence of the interaction with polyanions.

The exact mechanism by which HA affects the gene transfer process is still unknown. Interaction of HA with both polyplexes led to the formation of aggregates with a negative ζ potential. As the nature of the binding of nontargeted polyplexes with cells is mainly electrostatic, the reduced polyplex surface charge is likely to have limited the extent of the cellular uptake of both polyplex systems. Several studies indicate that the HA-induced changes in polyplex surface properties also affect the pathway involved in the uptake of the polyplexes by changing the interaction of the polyplexes with the different nonspecific internalizing cell-surface proteoglycans or by directing the uptake of the polyplexes to the specific internalizing HA-cell surface receptor (CD44).[32,34–36] The exact pathways responsible for the uptake and intracellular routing of HA associated polyplexes have not been solved to date, but it is highly likely that the subsequent intracellular rerouting of the polyplexes

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affects their transfection activity.[35–37] Another mechanism by which HA has been suggested to mediate changes in the overall gene transfer of polyplexes is by interfering with the process of endosomal destabilization.[31]

Conclusion

In this study, we evaluated polyplexes based on different biodegradable, methacrylamide polymers in the OVCAR-3 ovarian cancer mouse model. Polyplexes based on pHPMA−DMAE showed in vivo transfection activity which was similar to polyplexes based on the linear polymer PEI22.

Remarkably, pHPMA−DMAE- and PEI22-based polyplexes showed similar in vivo transfection activities, considering that their respective in vitro transfection activities differed 3−4 orders of magnitude, in favor of PEI22 polyplexes. The interaction of the polyplexes with hyaluronic acid, a polyanion present in the peritoneal fluid of ovarian cancer bearing mice, affected the polyplex properties of both carrier systems and impaired the transfection activity of PEI22 polyplexes but not of pHPMA−DMAE polyplexes. Therefore, differences of the polyplexes in HA-resistance may have contributed to the observed in vitro/in vivo discrepancy.

Polyplexes based on pHPMA−DMAE are biodegradable and HA resistant and do not show any cytotoxicity. These properties make pHPMA−DMAE-based polyplexes promising systems for use in ovarian cancer.

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Supporting Information Available: Bioluminescent image of a living mouse, bearing an i.p. OVCAR-3 xenograft and transfected with PEI22 polyplexes (N/P 6, 30 μg of DNA, i.p.). The image demonstrates how the luciferase protein is distributed throughout a major part of the peritoneal cavity. This material is available free of charge via the Internet at http://pubs.acs.org.

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