Polymer Side-Chain Degradation as a Tool to Control the Destabilization of Polyplexes

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Purpose. We purposed to design a cationic polymer that binds to pDNA to form polyplexes and that subsequently degrades within a few days at physiological pH and temperature, releasing the DNA in the cytosol of a cell.

Methods. We synthesized a new monomer carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester (abbreviated HPMA-DMAE) and the corresponding polymer. Hydrolysis of the carbonate ester of both the monomer and the polymer was investigated at 37°C. The DNA condensing properties of the pHPMA-DMAE was studied using dynamic light scattering (DLS) and zeta potential measurements. Degradation of the polyplexes at 37°C and pH 7.4 was monitored with DLS and gel electrophoresis. *In vitro* transfections were performed in COS-7 cell line.

Results. pHPMA-DMAE is able to condense DNA into small particles (110 nm) with a positive zeta potential. The half-life of the polymer and monomer at 37°C and pH 7.4 was around 10 h whereas at pH 5, the half-life was 380 h. In line with this, due to hydrolysis of the side groups, pHPMA-DMAE-based polyplexes dramatically increased in size at 37°C and pH 7.4 whereas at pH 5.0, only a very small increase was observed. Interestingly, intact DNA was released from the polyplexes after 48 h at pH 7.4 whereas all DNA remained bound to the polymer at pH 5.0. Polyplexes were able to transfect cells with minimal cytotoxicity if the endosomal membrane-disrupting peptide INF-7 was added to the polyplex formulation.

Conclusions. Degradation of the cationic side-chains of a polymer is a new tool for time-controlled release of DNA from polyplexes, preferably within the cytosol and/or nucleus.

KEY WORDS: biodegradation; gene delivery; polyplex.

INTRODUCTION

Gene therapy is considered to be a promising approach to treat life-threatening diseases. In order to introduce successfully foreign DNA into a cell, extracellular degradation of DNA has to be prevented and cellular uptake has to occur. To achieve this, both viral and nonviral carrier systems have been developed over the years. Although viral systems ("vectors") are highly efficient in introducing DNA into cells (1,2), they possess some serious disadvantages, such as the possible induction of immune responses and problems with pharmaceutical-grade large-scale productions. Therefore, as an alternative for viral vectors, attention is focused on the design of nonviral carriers. These include cationic polymers such as poly-L-lysine (pLL), poly(ethylene imine) (pEI), and poly(dimethylamino methacrylate) (pDMAEMA) or cationic lipids such as DOTAP or DOPE (3–6). However, the current nonviral carriers are less efficient in their transfection activity than viral vectors. It is commonly accepted that polymer–DNA complexes (also called polyplexes) are taken up by cells by endocytosis (7). DNA has to be protected against degradation inside the endosomes/lysosomes which is established as long as the polyplexes stay intact in these cellular compartments. Although some cationic polymers as such are thought to be able to destabilize endosomes [e.g., pEI (8), polyamidoamine dendrimers (9), or pDMAEMA (10)], endosomal escape can be promoted using specific compounds [e.g., Gala- (11) and INF-peptides (12,13) or poly(propylacrylic acid) (14)]. Once escaped from the endosome, DNA has to dissociate from the polymer and be transported into the nucleus for transcription. Dissociation of polyplexes may occur by anionic compounds (e.g., proteins or RNA) present in the cytosol (5,15). Alternatively, dissociation of the DNA from the polymer can be achieved by polymer degradation. Poly(4-hydroxy-L-proline ester) has been studied as one of the first water-soluble, degrading gene delivery polymers (16,17). This polymer showed a rapid degradation in the first 2 h at 37°C and pH 7.0, after which degradation slowed down. Importantly, the degradation of the polymer was retarded when it was complexed with DNA. A similar degradation behavior was found for poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) (18,19). Recently, degradable pEIs were designed by linking low-molecular-weight pEI blocks with oligo(L-lactic acid-co-succinic acid) (20) or poly(ethylene glycol) (21) via degradable bonds. However, the degradation time was rather slow for the first polymer, taking weeks up to months, and degradation of the PEG-containing copolymer is not reported yet. Recently, Luten *et al.* reported on a new class of cationic polymers for gene delivery: polyphosphazenes (22). The half-lifes of the polymers were of the order weeks. Cellular uptake of lipo/polyplexes and escape from the endosome into the cytoplasm takes a number of hours (23). This means that the polyplexes should be stable for a couple of hours after administration and also at pH 5 (inside the endosome), thereby protecting DNA against degradation by endosomal/lysosomal DNases. Given the time between administration and intracellular presence, the ideal degradable carrier is stable at pH 5 but degrades after a few hours at pH 7.4. The biodegradable polymers investigated so far rely on the (complete) degradation of the polymer backbone, and the degradation of these polymers is rather slow. The alternative approach we have chosen in this study is to design a polymer with degradable cationic side groups, meaning that the backbone stays intact but the plasmid condensing side-chains are removed in time. The condensing capacity of the polymer, which relies on the multivalency of the interaction with the plasmid, thereby decreases, and the plasmid will be released and become available for intracellular transport. The polymer that has been intensively studied for gene delivery in our research group is pDMAEMA (10), which carries an ester bond between the polymer backbone and each cationic side group (Fig. 1). However, unlike the intrinsic hydrolytical sensitivity of an ester group, it was demonstrated that this polymer is completely stable at any tested pH, probably because of the inability of water to access the ester bond, being too

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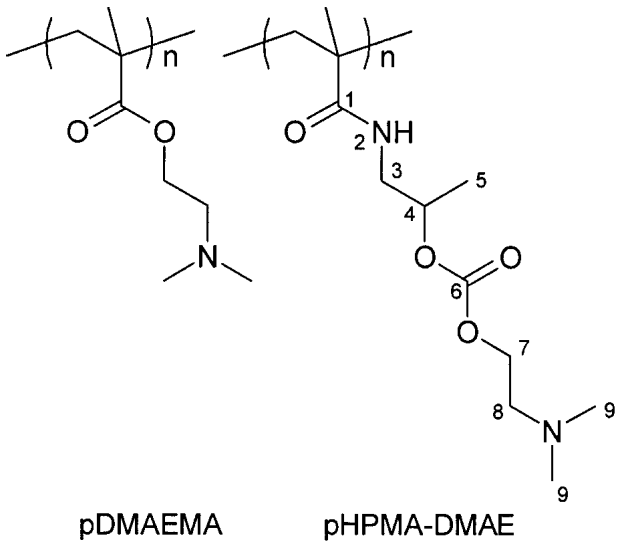

Fig. 1. Structural formulas of the polymers used in this study.

close to the hydrophobic polymer backbone (24). Therefore, we designed a similar polymer, containing a degradable bond between the cationic group and the polymer backbone (Fig. 1). We chose a carbonate ester, whose degradation rate is higher than a normal ester and therefore favorable (25). The backbone is derived from poly-2-hydroxypropyl methacrylamide (pHPMA), which is known to have no polymer-related toxicity (26,27). The cationic group of the polymer is the same as for pDMAEMA, the dimethyl amino group, which is partially protonated at physiological pH, thus ensuring DNA binding and condensation capability (10).

This study reports on the synthesis of the monomer and polymer; the pH-dependent degradation of the monomer, polymer, and polyplexes; and the transfection activity of polyplexes.

MATERIALS AND METHODS

Materials

The following materials were used as received: D,L-1-amino-2-propanol 99+ % (Acros), *N,N*-dimethylaminoethanol (DMAE, Aldrich, Zwijndrecht, The Netherlands), 1,1'-carbonyldiimidazole (CDI, Acros, Geel, Belgium). Methacryloyl chloride of purity ≥ 97% (Fluka, Zwijndrecht, The Netherlands) was freshly distilled before use. pDMAEMA (M_n = 92 kg/mol) was synthesized via radical polymerization (10). The plasmid pCMVLacZ, containing a bacterial LacZ gene preceded by a nuclear localization signal under control of a cytomegalovirus (CMV) promoter, was purchased from Sanvertech (Heerhugowaard, The Netherlands). INF-7, a 24 amino acid containing peptide with fusogenic activity derived from the influenza virus, was synthesized via standard Fmoc solid-phase synthesis (12). HPMA was synthesized according to Oupicky *et al.* (28).

Synthesis of Carbonic Acid 2-Dimethylamino-Ethyl Ester 1-Methyl-2-(2-Methacryloylamino)-Ethyl Ester and Corresponding Polymer [(p)HPMA-DMAE]

A dry round-bottom flask was loaded with 1,1'-carbonyldiimidazole (6.84 g, 45.0 mmol, CDI) and 50 ml dichloromethane. To this suspension 2.50 ml *N,N*'-dimethylaminoethanol (2.22 g, 24.9 mmol, DMAE) was added dropwise. During addition, the CDI dissolved and the obtained clear solution was stirred at room temperature for 1 h. Next, this solution was washed with water (three times 20 ml), dried over magnesium sulfate, and the solvent was evaporated under reduced pressure, yielding DMAE-CI as a colorless liquid (3.12 g, 68%).

NMR spectra were recorded on a Varian G-300 300 MHz spectrometer (Varian, Palo Alto, CA, USA).

NMR ($CDCl_3$, δ in ppm): ^{1}H: 2.29 (s, 6H, N($C\underline{H}_3$)$_2$), 2.68 (t, 2H, $C\underline{H}_2$N), 4.48 (t, 2H, O$C\underline{H}_2$), 7.04 (s, 1H, NC$\underline{H}$C$\underline{H}$N), 7.40 (s, 1H, NC$\underline{H}$CHN), 8.12 (s, 1H, NC$\underline{H}$N). ^{13}C: 45.7 (N($\underline{C}H_3$)$_2$), 57.5 ($\underline{C}H_2$N), 65.9 (O$\underline{C}H_2$), 117.1 (N$\underline{C}$HCHN and NCH$\underline{C}$HN), 130.6 (N$\underline{C}$HN).

DMAE-CI (3.12g, 17.0 mmol) was dissolved in 30 ml dichloromethane and 1.83 g HPMA (12.8 mmol, 0.75 equivalents) dissolved in 20 ml dichloromethane was added. Hydroquinone monomethyl ether (10 mg) was added to prevent premature polymerization. This solution was put under a nitrogen atmosphere and stirred at room temperature for 5 days. The solvent was removed under reduced pressure, yielding an oil (5.11 g, mixture of imidazole, unreacted DMAE-CI, and HPMA-DMAE).

NMR ($CDCl_3$, δ in ppm): ^{1}H: 6.15 (bs, 1H, CON$\underline{H}$C$\underline{H}_2$), 5.63 (s, 1H, $\underline{H}_2$C=C), 5.34 (s, 1H, $\underline{H}_2$C=C), 4.86 [m, 1H, $CH_2C\underline{H}$(CH3)O], 4.20 (m, 2H, O$C\underline{H}_2C\underline{H}_2$), 3.7–3.3 [m, 2H, $C\underline{H}_2$CH(CH3)O], 2.48 (t, 2H, O$C\underline{H}_2C\underline{H}_2$), 2.22 [s, 6H, N($C\underline{H}_3$)$_2$], 1.97 [s, 3H, $H_2C=C(C\underline{H}_3)$], 1.27 [d, 3H, $CH_2CH(C\underline{H}3)$O]. ^{13}C: 168.8 ($\underline{C}$=O), 154.7 ($\underline{C}$=O), 135.2 ($H_2C=\underline{C}$), 121.7 ($H_2\underline{C}=C$), 74.1 ($\underline{C}$H), 65.1 (O$\underline{C}H_2$), 57.4 ($\underline{C}H_2$N), 45.3 [N($\underline{C}H_3$)$_2$], 43.8 (NH$\underline{C}H_2$), 18.5 (CH$\underline{C}H_3$), 17.3 ($C\underline{C}H_3$).

pHPMA-DMAE was synthesized by radical polymerization under a nitrogen atmosphere as follows: approximately 1 g of the crude monomer was dissolved in 4 ml of 1 M aqueous hydrochloric acid solution, and the pH was adjusted to 5. Polymerization was carried out under shaking conditions at 70°C with ammonium peroxodisulfate as initiator (M/I = 25). After 20 h, the polymerization mixture was cooled down to room temperature and transferred into a dialysis tube (MWCO 3.5 kDa). After extensive dialysis against an NH_4Ac buffer of pH 5.0 (10 mM, last step 5 mM) at 4°C, the polymer was collected after freeze drying. Molecular weight of the polymer relative to dextran standards (Fluka) was determined by gel permeation chromatography (GPC) (10).

Hydrolysis of the Monomer

Monomer (60 mg, crude mixture) was dissolved in 100 ml of the appropriate buffer. Buffers used were acetic acid at pH 5.0 and HEPES at pH 7.4 (concentration buffer 10 mM, ionic strength adjusted to 150 mM with NaCl). The solutions were incubated at 37°C, and samples were periodically withdrawn from these solutions and immediately analyzed with RP-HPLC.

The RP-HPLC system consisted of a Waters pump model 515 (Waters Associates, Milford, MA, USA), Spark Marathon Basic$^+$ injector (Spark, Emmen, The Netherlands) and a LKB 2151 UV detector (LKB, Roosendaal, The Netherlands) set at 210 nm. The columns used were a Merck LiChrosphere 100 (Merck, Darmstadt, Germany) RP-18 column (5 μm, 125 × 4 mm i.d.) and a RP-18 guard column (4 × 4 mm). The mobile phase consisted of a water–acetonitrile

95/5 (w/w) solution completed with 10 mM triethylamine and brought to pH 2 with perchloric acid. The flow rate was 1 ml/min; injection volume was 20 μl.

Hydrolysis of the Polymer

Hydrolysis of the polymer was followed in time with ^{1}H-NMR in D_2O buffered at pD 7.4 with NaH_2PO_4/Na_2HPO_4; ionic strength adjusted to 150 mM with NaCl at 37°C in a Varian Gemini 500 MHz NMR apparatus. Each hour a proton NMR spectrum was run, and the peak area of the $CH_2C\underline{H}(CH_3)O$ (4 in Fig. 1) resonance was plotted in time.

Physical Characteristics of pHPMA-DMAE-Based Polyplexes

The physical characteristics of pHPMA-DMAE-based polyplexes were investigated as a function of the polymer-nitrogen to DNA-phosphate (N/P) ratio. Plasmid DNA was diluted in 20 mM HEPES buffer, pH 7.4, to a concentration of 75 μg/ml. A stock solution of the polymer (5 mg/ml) was diluted in the same buffer to various concentrations (7–450 μg/ml). Polyplexes were made by the addition of 700 μl of a polymer solution to 175 μl DNA solution, mixed thoroughly and incubated for 30 min at room temperature. The *Z*-average diameters of the formed polyplexes were determined via dynamic light scattering (DLS) at 25°C, and zeta-potential measurements were carried out as previously described (10).

Polyplex Destabilization Studied with DLS

Polyplexes of pHPMA-DMAE and plasmid DNA were made at a N/P ratio of 5.4, either in 10 mM HEPES pH 7.4 or 10 mM acetic acid pH 5, ionic strength adjusted to 150 mM with NaCl. The size of these polyplexes was measured every half hour with dynamic light scattering at 37°C for approximately 15 h. As a control, pDMAEMA-based polyplexes (N/P = 6) in HEPES buffer were measured.

Polyplex Destabilization Studied with Agarose Gel Electrophoresis

Polyplexes of pHPMA-DMAE and plasmid DNA were made by mixing 10 μl of plasmid solution, 200 μg/ml, with 40 μl of pHPMA-DMAE solution, 200 μg/ml, corresponding with a N/P ratio of 5.4 and incubated for 30 min at room temperature. This was done in 10 mM HEPES pH 7.4 or 10 mM sodium acetate pH 5; ionic strength adjusted to 150 mM with NaCl. As a control, pDMAEMA-based polyplexes at pH 7.4 were also investigated. These polyplex dispersions were subsequently incubated at 37°C for 0, 2, 4, 7, 24, or 48 h. After cooling down to room temperature, 10 μl of the corresponding buffer and 3 μl of sample buffer (0.4% w/v bromophenol blue, 10 mM EDTA, 50% v/v glycerol in water) were added to 20 μl of each polyplex dispersion. Thirty microliters of these solutions were applied to a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide and were run at 100 V (15).

Transfection Studies

Transfection studies were done in COS-7 cells using the plasmid pCMV*LacZ* as reporter gene as previously described (10,15). In brief, 96-well plates were seeded with cells at a density of 3×10^4 /cm^2 24 h before transfection. At the day of transfection, polyplexes were prepared as follows: 150 μl of polymer solution (various concentrations) was added to 50 μl of plasmid solution (50 μg/ml), and after incubation for 30 min at room temperature, 50 μl of either buffer or INF-7 solution (150 μg/ml) was added, and the polyplexes were incubated for another 15 min before addition to the cells. After rinsing the cells with HBS, 100 μl of polyplex dispersion and 100 μl of culture (with or without 10% serum) 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. As a reference, pDMAEMA polyplexes prepared at the same DNA concentration and an N/P ratio of 6 were used. The transfection activity of this polyplex formulation was set at one.

RESULTS AND DISCUSSION

Synthesis and Characterization of (p)HPMA-DMAE.

Synthesis of the monomer was performed in two steps. In the first step *N'N'*-dimethylaminoethanol (DMAE) was activated by 1,1'-carbonyldiimidazole in a good yield (68%) and good purity (>97% by NMR). In the second step, the activated alcohol was reacted with *N*-(2-hydroxypropyl) methacrylamide (HPMA). A kinetic study showed that when a slight excess (~30 mol%) of activated alcohol was used, no unreacted HPMA could be detected by proton NMR after 5 days of reaction at room temperature. Removal of the solvent resulted in a mixture of the desired product (HPMA-DMAE) as well as unreacted activated DMAE and imidazole. Purification of HPMA-DMAE from the reaction mixture was difficult to achieve (premature degradation of this compound; low yields). The crude mixture was therefore directly polymerized because the impurities did not interfere with the radical polymerization and could easily be removed by the dialysis procedure applied to purify and isolate the polymer.

Radical polymerization of the monomer was performed in an aqueous 1 M HCl solution (with pH adjusted to pH 5 if necessary to prevent degradation of the monomer) with good yield (75%). The weight-average molecular weight (M_w) of the polymer was 160 kDa and the number-average molecular weight (M_n) was 20 kDa.

Hydrolysis of Monomer and Polymer

The hydrolysis of the monomer at 37°C was followed with RP-HPLC. With the HPLC method used, both the remaining HPMA-DMAE as well as one of the hydrolysis products (HPMA) could easily be detected and quantified. The relative peak areas of the HPMA-DMAE and HPMA as a function of time and at pH 7.4 and 5 are depicted in Fig. 2 (note the different timescales of the *x* axes of the graphs). The k_{obs} for the hydrolysis of the monomer was calculated by plotting the natural logarithm of the peak areas divided by the peak area at time point zero vs. the time (see insets in the figures; the calculated k_{obs} at pH 5 and 7.4 are 5.0×10^{-7} s^{-1} [(half-life, 380 h) and 1.7×10^{-4} s^{-1} (half-life, 9.6 h), respec-

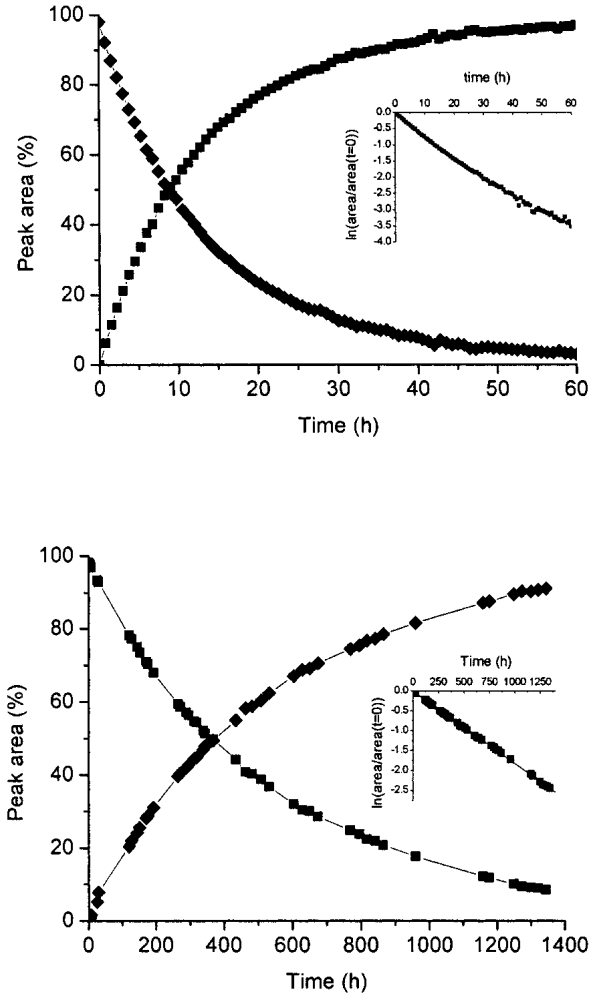

Fig. 2. (♦) Hydrolysis of the HPMA-DMAE monomer and (■) formation of HPMA in time (37°C) at pH 7.4 (top) and at pH 5.0 (bottom). The insets show the natural logarithm of the peak areas divided by the peak area at time point zero vs. the time.

tively]. The hydrolysis of pHPMA-DMAE was measured with ^{1}H-NMR by following the integral of the $CH_2C\underline{H}(CH_3)O$ peak from HPMA-DMAE (4 in Fig. 1), which shifts from 4.8 to 3.9 ppm when the DMAE part is removed by hydrolysis, in time (Fig. 3). The estimated half-life of the polymer is ~12 h, which corresponds well with the half-life of the monomer under the same condition. This is much faster than other reported polymers like pEI linked via oligo(L-lactic acid-co-succinic acid) (20) or polyphosphazenes (22).

Biophysical Characterization of p(HPMA-DMAE)-Based Polyplexes

The DNA binding and condensation properties of p(HPMA-DMAE) were investigated by DLS and zeta potential measurements as a function of the polymer to DNA ratio. Figure 4 shows that when an excess of the polymer was added (N/P > 4), the polymer was able to condense DNA into small particles with a size of approximately 110 nm and a positive zeta potential of approximately +19 mV. Neutral aggregates were formed at an N/P ratio between 2 and 4, and negatively charged polyplexes were formed at a ratio of 1. This behavior

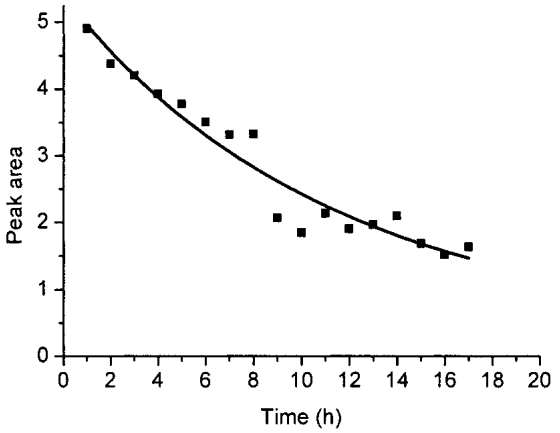

Fig. 3. Hydrolysis of p(HPMA-DMAE) in time at pH 7.4 and 37°C. Integral of the ^{1}H-NMR peak at 4.8 ppm is shown on the *y* axis.

is comparable to polyplexes composed of DNA and other cationic polymers (13,22,29).

Polyplex Destabilization Studied with DLS

The destabilization of p(HPMA-DMAE)-based polyplexes was investigated at pH 5.0 and pH 7.4 at 37°C by dynamic light scattering. Figure 5 depicts the particle size of the polyplexes as a function of time. While the size of polyplexes based on p(HPMA-DMAE) at pH 5.0 and of pDMAEMA polyplexes at pH 7.4 was constant over the investigated time period, the size of p(HPMA-DMAE)-based polyplexes at pH 7.4 increased dramatically. This indicates that the side chains of p(HPMA-DMAE) indeed are removed upon hydrolysis, even in the polyplexes. Due to the hydrolysis, the charge density of the polymer decreases in time, resulting in decreasing binding/condensing capabilities of the polymer. As the result, the polyplexes swell/aggregate in time (Fig. 4). The time period in which aggregation of the polyplexes occurs is of the same order of magnitude as monomer/polymer hydrolysis (compare Fig. 5 with Figs. 2 and 3).

Polyplex Destabilization Studied with Agarose Gel Electrophoresis

Polyplexes incubated at 37°C for different time periods were subjected to gel electrophoresis to investigate whether

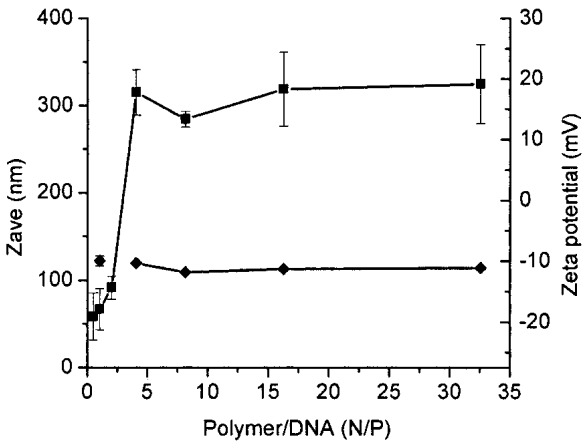

Fig. 4. (♦) Particle size and (■) zeta potential of p(HPMA-DMAE)-based polyplexes as a function of the N/P ratio.

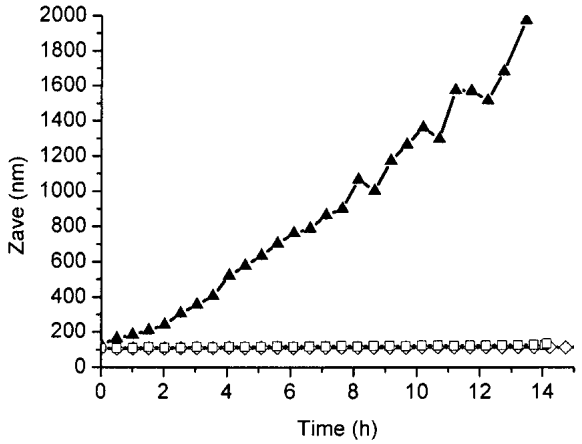

Fig. 5. The size of polyplexes based on p(HPMA-DMAE) incubated at (▲) pH 7.4 and (□) pH 5.0 or pDMAEMA at pH 7.4 (◇).

the DNA was released from polyplexes by hydrolysis of p(HPMA-DMAE). Figure 6 shows that for pDMAEMA at pH 7.4 or p(HPMA-DMAE) at pH 5.0, all DNA remained in the starting slots indicating that the DNA remained complexed with the polymers. At pH 7.4 and 0 h and 4 h incubation, all DNA was present in the starting slot, indicating that the polymer was still complexed with DNA. At 7 and 24 h of incubation, no fluorescence signal could be detected. This can probably be attributed to the presence of aggregates (see Fig. 5), which are not accessible for ethidium bromide, as has also been observed for pEI-DNA aggregates (30). Interestingly, after 48 h incubation, DNA was released in its intact form from the p(HPMA-DMAE)-based polyplexes, indicating that the polymer was hydrolyzed to such an extent that dissociation of the polyplexes had occurred. DNA release takes place between approximately 2–4 half-lifes; in that time, at least 75% of the side chains have degraded.

Both the DLS and gel electrophoresis experiments indicate that at physiological pH and temperature, the hydrolysis of the polymer complexed with DNA is relatively fast whereas at pH 5, the complexes are stable. In the literature, it was shown that when DNA is complexed with a polymer, degradation by DNases is slowed down considerably (15,31). As DNA stays complexed with the polymer inside endosomes/lysosomes (pH 5), degradation by DNases is minimized, while once inside the cytoplasm DNA will be released via hydrolysis of the polymer cationic side-chains. However, this hydrolysis is not so fast that DNA is released before cellular uptake can take place. Both experiments indicate that hydrolysis of the polymer is not or is hardly influenced by complexation with DNA. This is in contrast to other degradable polymers like PAGA, where degradation was influenced by complexation with DNA (16).

Transfection Efficiency and Cytotoxicity of p(HPMA-DMAE)-Based Polyplexes

The transfection activity of pHPMA-DMAE-based polyplexes was investigated in COS-7 cells. When serum proteins were present during transfection, no transfection activity was found (data not shown). A low transfection activity of poly/lipoplexes in the presence of serum proteins has been reported before, and this was ascribed to aggregation and/or destabilization of the poly/lipoplex due to proteins (32). This can be prevented by pegylation of the polymer or the polyplexes, as has been shown for pDMAEMA (33). However, this was not the intention of this study. Therefore, serum proteins were absent in further transfection experiments.

Figure 7 shows the transfection of COS-7 cells and cytotoxicity of p(HPMA-DMAE)-based polyplexes as a function of the polymer to DNA ratio in the absence or presence of the membrane-disrupting peptide INF-7. As a reference, pDMAEMA was used as a transfection agent, and its highest transfection efficiency was set to 1. p(HPMA-DMAE)-based polyplexes in the absence of INF-7 are not toxic to cells at any of the tested concentrations and are able to transfect cells to some extent, but only at rather high polymer to DNA ratios. This behavior has been seen for other polymers and is probably related to the low toxicity of the polymer (13,22). Interestingly, when the membrane-destabilizing peptide INF-7 was added to the polyplex dispersion, a drastic increase in the transfection activity was observed. This likely indicates that p(HPMA-DMAE) polyplexes do not escape from the endosome, as previously reported for other polymers (12,13). Addition of the INF-7 peptide to pDMAEMA-based polyplexes

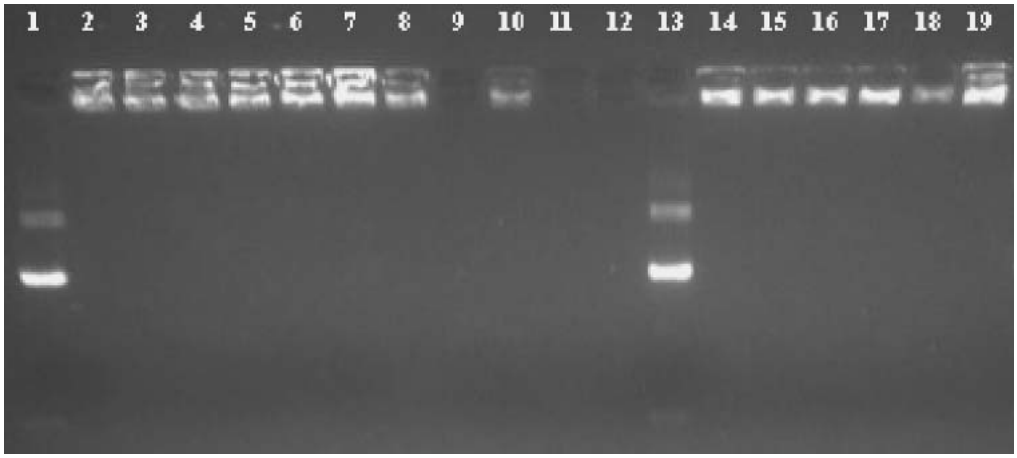

Fig. 6. Gel electrophoresis of polyplexes incubated at 37°C for 0, 2, 4, 7, 24, or 48 h. Lane 1, free DNA; lanes 2–7, pDMAEMA-based polyplexes, pH 7.4; lanes 8–13, p(HPMA-DMAE)-based polyplexes pH 7.4 [lane 9 (t = 2 h) is empty]; lanes 14–19, p(HPMA-DMAE)-based polyplexes pH 5.0.

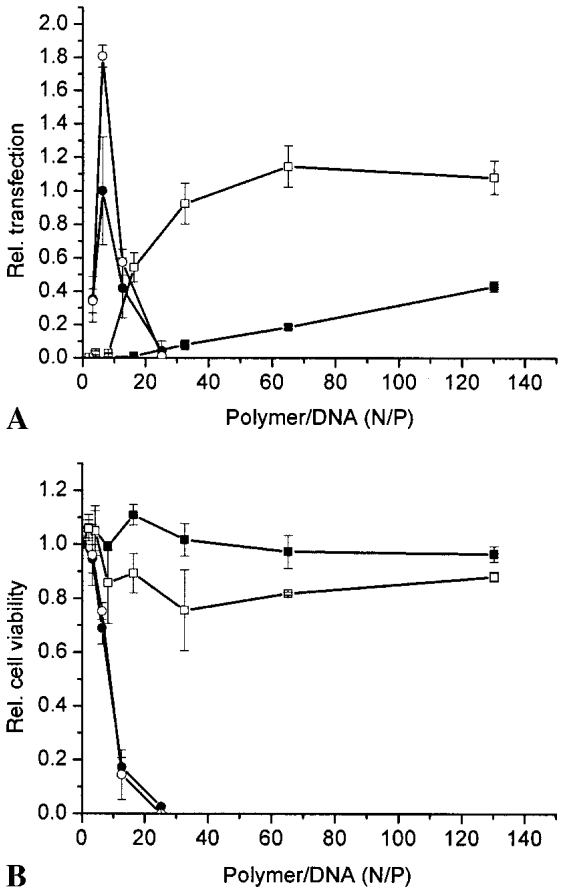

Fig. 7. (A) Transfection efficiency and (B) cytotoxicity of polyplexes based on p(HPMA-DMAE) (■) without and (□) with the membrane-disrupting peptide INF-7. As a reference, pDMAEMA (●) without and (○) with the peptide was used. Cells are grown for 48 h after transfection.

also increased the transfection efficiency, however, the increase was less dramatic than for p(HPMA-DMAE)-based polyplexes [1.8-fold increase for pDMAEMA polyplexes vs. 6.2-fold increase for p(HPMA-DMAE) polyplexes]. This can be ascribed to the superior intrinsic endosomal escape capability of pDMAEMA polyplexes. For pDMAEMA polyplexes, the capacity of INF to break down the endosomal barrier adds little to the transfection efficiency. p(HPMA-DMAE) polyplexes coated with the INF peptide showed some minor cytotoxicity, but this toxicity is independent of the polymer to DNA ratio (Fig. 7) and thus probably caused by the peptide and not by the polymer. For pDMAEMA polyplexes, coating with INF-7 does not influence the (substantial) toxicity of the polymer.

Although cytosolic delivery of released DNA is likely enhanced for pHPMA-DMAE polyplexes (after endosomal escape via the INF peptide) because of the hydrolysis of the cationic side-chains and the subsequent disintegration of the polyplexes, the transfection efficiency is comparable to polyplexes based on the nondegradable polymer pDMAEMA. It is possible that one of the next barriers, like nuclear transport or nuclear uptake, is limiting the transfection process, and therefore protein expression is not enhanced for pHPMA-DMAE-based polymers. Passage of these barriers may be facilitated for nonviral vectors by improving the cellular trafficking of the DNA toward the nucleus via nuclear localization signal (NLS) peptides (34,35). Transfection with NLS containing DNA is currently under investigation.

CONCLUSIONS

We have synthesized a new gene delivery polymer (pHPMA-DMAE, Fig. 1) with hydrolyzable cationic side-groups. It was shown that polyplexes based on this polymer were stable at pH 5, but showed release of intact DNA within 48 h after incubation at pH 37°C and pH 7.4. Polyplexes based on this polymer were able to transfect cells efficiently when a membrane-destabilizing peptide was present while not being toxic toward the cells. Thus, polymer side-chain degradation is a new tool for controlled intracellular destabilization of polyplexes. Future work will focus on nuclear targeting of the intracellularly released DNA to further enhance transfection efficiency and *in vivo* application of the new polymer.

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