Association and Dissociation Characteristics of Polymer/DNA Complexes Used for Gene Delivery

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Purpose. The DNA association/dissociation properties of water-soluble cationic methacrylate polymers with closely related structures (poly(2-dimethylamino)ethyl methacrylate) [p(DMAEMA)], poly(2-(trimethylamino)ethyl methacrylate chloride) [p(TMAEMA)]) and the frequently used transfectant poly(L-lysine) were studied to gain a better insight into their transfection characteristics.

Methods. Association of DNA with different polymers and dissociation of the complexes, achieved by adding an excess of anionic polymers or salt, were studied by using spectroscopic techniques (fluorescence, circular dichroism (CD)), agarose gel electrophoresis and an enzymatic assay (DNase I treatment). The transfection efficiency of the polyplexes was evaluated in tissue culture with OVCAR-3 cells.

Results. Plasmid DNA complexed with either poly(L-lysine) or p(DMAEMA) was protected against digestion by DNase I. Fluorescence and CD spectroscopy as well as gel electrophoresis revealed that p(DMAEMA) with a relatively high molecular weight and poly(L-lysine) have similar DNA association/dissociation characteristics. Therefore, differences in transfection potential of the polyplexes cannot be ascribed to differences in binding characteristics, but are probably caused by other factors. As compared with the other polymers, p(TMAEMA) has a high affinity for DNA as was concluded from the observation that poly(aspartic acid) was unable to fully dissociate complexes containing this polymer. This fact might very well explain the low transfection efficiency of these polyplexes. p(DMAEMA) with a relatively low molecular weight probably has a low affinity for DNA, which might explain both the formation of DNA aggregates (Ψ-DNA) and the low transfection potential obtained when using this polymer.

Conclusions. DNA association/dissociation studies shed light on the preferred characteristics of polymeric transfectants.

KEY WORDS: gene delivery; poly(L-lysine); DNA interaction; plasmid; p(DMAEMA).

INTRODUCTION

Cationic polymers have been described as DNA-condensing agents and have promising characteristics as non-viral gene delivery systems (1–3). The cationic polymer that forms the basis for this self-assembling system interacts electrostatically with the phosphate groups of DNA to form stable nanoparticles, also called polyplexes (4). In recent studies of our group we demonstrated that p(DMAEMA) and its copolymers with triEGMA and NVP have a very favorable transfection efficiency/toxicity ratio as compared with 'standard' transfectants (e.g., lipofectin, p(Lys), DEAE, dextran) (5). Interestingly, we found large differences among the transfection properties of methacrylate/methacrylamide polymers with structures closely related to p(DMAEMA) (6).

To achieve transfection, the plasmid has to be delivered into the nucleus, which requires cellular uptake of the polyplex, most likely by endocytosis, endosomal escape, dissociation of the polyplex and transport to the nucleus. In a recent paper of our group it has been hypothesized that the superior efficiency of p(DMAEMA) as compared with other methacrylated polymers is due to (a) its ability to destabilize endosomes because of the presence of tertiary amines with a pK_a around 7.5 that act as a proton sponge (7) and (b) an easy dissociation of the polyplex once present in the cytosol and/or nucleus. It is essential for the DNA to be stable during every step of the transfection process (e.g., protected against DNase degradation) and that its structure is preserved after release from the polymer.

This study is aimed to give a detailed characterization of the interaction between DNA and cationic polymers, in particular the association and dissociation processes. The relevance of these characteristics for in vitro transfection efficiency is discussed.

MATERIALS AND METHODS

Materials

2-(trimethylamino)ethyl methacrylate chloride (TMAEMA, Aldrich), ammonium peroxodisulfate (APS, Fluka, Bornem, Belgium), dextran standards (Fluka) and N,N-diethylformamide (DMF, Acros, Zwijndrecht, The Netherlands) were used as received. Water purified by reversed osmosis was applied throughout the study.

2-(Dimethylamino)ethyl methacrylate (DMAEMA, Fluka) was purified by distillation under reduced pressure before use.

Ortho-nitrophenyl-β-D-galactopyranoside (ONPG), sodium 3′-[1-(phenyl aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT), β-galactosidase and poly-L-aspartic acid (molecular weight between 5,000–15,000 D) were obtained from Sigma (Zwijndrecht, The Netherlands). 4-(2-hydroxy ethyl)-1-piperazine-ethanesulfonic acid (HEPES) and ehtylenediamine tetraacetic acid, disodium salt (EDTA) were obtained from Acros. Dextran sulfate (molecular weight 40,000 D) was obtained from ICN (Zoetermeer, The Netherlands). Acridine orange was obtained from Molecular Probes (Eugene, OR). DNase I was obtained from Pharmacia Biotech (Roosendaal, The Netherlands). All other chemicals used were of analytical grade.

Synthesis and Characterization of Polymers

P(DMAEMA) with a relatively low molecular weight (LMW, Mn 2.5 kD, Mw 4 kD) and with a relatively high molecular weight (HMW, Mn 45 kD, Mw 360 kD) and

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ABBREVIATIONS p(DMAEMA), poly(2-(dimethylamino)ethyl methacrylate; p(TMAEMA), poly(2-(trimethylamino)ethyl methacrylate chloride); p(Lys), poly(L-lysine); NVP, N-vinyl pyrrolidone; triEGMA, ethoxytriethylene glycol methacrylate; p(Asp), poly(L-aspartic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; TAE, Tris acetate EDTA buffer; LMW, low molecular weight; HMW, high molecular weight.

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p(TMAEMA) (Mn 72 kD, Mw 845 kD) were synthesized by radical polymerization of the corresponding monomers with APS as initiator, as described in detail previously (2,6). P(Lys) (Mw 120 kD) was synthesized via multiple step synthesis from L-lysine HCl according to procedures previously described (2).

Preparation of Plasmid

The plasmid pCMV-LacZ containing a bacterial LacZ gene preceded by a nuclear localization signal under control of a CMV promoter was used (8). The plasmid was amplified and purified as reported before (9).

CD Measurements

Stock solutions of all polymers and plasmid DNA used were prepared in 20 mM Hepes, pH 7.4 (1–5 mg/mL). Polymer/DNA complexes were prepared by mixing solutions of known concentration. The final DNA concentration was 13.2 μg/mL ($4 \cdot 10^{-5}$ M based on the number of bases) for all the ratios used. Spectra were recorded at 25°C in quartz cells of 1 cm path length with a spectropolarimeter J-600 (Mod. 7800, Jasco, Tokio, Japan). The scan speed was fixed at 20 nm/min and the step resolution was 2 nm. Spectra were recorded from 350 to 220 nm and $\Delta\lambda$ was calculated as the difference between the wavelength values where the maximum (positive or negative) molar CD ($\Delta\varepsilon$) of polyplexes and free DNA occurred. The difference between the maximum (positive or negative) molar CD of polyplexes and free DNA (%$\Delta(\Delta\varepsilon)$) was calculated as: $[(\Delta\varepsilon_{Polyplexes} - \Delta\varepsilon_{DNA})/\Delta\varepsilon_{DNA}] \times 100\%$. For dissociation studies, increasing amounts of 3 M NaCl or 5 mg/mL dextran sulfate were added to polyplexes.

Fluorescence Measurements

Fluorescence values (λ_{ex} 500 ± 2.5 nm and λ_{em} 525 ± 2.5 nm) of 2 μg/mL acridine orange were measured on an LS50B luminescence spectrometer (Perkin Elmer, Norwalk, CT) before (F_0) and after the addition of DNA (F_{DNA}; final DNA concentration = 10 μg/mL), and after the addition of different amounts of polymer in HBS (Hepes buffered saline, 20 mM Hepes, 150 mM NaCl, pH 7.4) to the DNA solution (F_{pol}) under stirring conditions at 25°C. The percentage of DNA phosphate groups that interacted with the fluorescent probe, was calculated by $(F_{pol} - F_0)/(F_{DNA} - F_0) \times 100\%$. Dissociation of polyplexes was studied by measuring the fluorescence once a plateau value was reached after addition of dextran sulfate or p(Asp) (final concentrations 100 μg/mL).

Dissociation Studies Monitored by Agarose Gel Electrophoresis

The exchange reaction of complexed DNA with p(Asp) (10) was carried out as follows: polyplexes of p(DMAEMA), p(TMAEMA) or p(Lys) at increasing polymer/DNA ratios, ranging from 0.2/1 to 3/1 (w/w), were formed by mixing solutions of known concentration of DNA and polymer in 20 mM HEPES buffer (pH 7.4) and incubating at room temperature for 30 min (final DNA concentration 80 μg/mL). Dissociation of polyplexes was achieved by adding an excess of p(Asp) to the incubated samples (12.5 μg p(Asp)/μg DNA). Polyplex samples, whether or not treated with p(Asp), were analyzed by electrophoresis in a 0.7% agarose gel containing 0.5 μg/mL ethidium bromide, in TAE buffer (pH 7.4). Visualization of the DNA present in the gels was performed by exposure to UV light. The marker lambda-Hind III was used as control.

Nuclease Resistance of p(DMAEMA)/DNA Complexes

Polyplexes of p(DMAEMA), p(TMAEMA) or p(Lys) at increasing polymer/DNA ratios were formed in 20 mM HEPES and 10 mM $MgCl_2$ (pH 7.4) (final DNA concentration 10 μg/mL). After 30 min of complexation, the polyplexes were incubated with DNase I (0.25 U/μg DNA) for 10 min at 37°C. The activity of DNase I was stopped by addition of an excess of EDTA (final concentration 0.15 M). Complexes were dissociated by adding NaCl to a final concentration of 1 M and an excess p(Asp) (12.5 μg/μg DNA), and the samples were analyzed by gel electrophoresis as described above. Free DNA without any treatment was used as a control.

Cell Culture

The human ovarian cancer cell line NIH (OVCAR-3) was originally obtained from Dr. Hamilton (National Cancer Institute, Bethesda, MD, USA) (11). OVCAR-3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine (2 mM), L-glucose (4.5 g/L), penicillin (100 IU/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL).

Transfection Studies

Transfection experiments were performed with OVCAR-3 cells by using the plasmid pCMV-LacZ as reporter gene essentially as described before (5,9). In brief, cells were seeded at a concentration of $1.1 \cdot 10^4$/well in 96-well plates 24 h before transfection. At the day of transfection, cells were rinsed and overlaid with 100 μl of fresh culture medium. Polyplexes were prepared by adding 200 μL of polymer solution (concentrations ranging from 18.5 to 150 μg/mL) to 50 μL of plasmid solution (50 μg/mL). After 30 min of incubation of the polyplexes, cells were overlaid with 100 μL of polyplex dispersion per well and further incubated for 1 h at 37°C. After removal of the transfection complexes, 100 μl of fresh culture medium was added, and the cells were further cultured for about 44 hours. All transfection experiments were performed in two identical series on two separate plates. One series was tested for reporter gene expression (β-galactosidase) by using a modification of the ONPG assay (12) (ONPG solution at 0.68 μg/mL and measuring extinction in time at 450 nm during 30–60 min). This assay is based on the conversion of ONPG by β-galactosidase in a coloured product. The second series was used to determine the cytotoxic effect of polyplexes, by measuring the number of viable cells with an XTT colorimetric assay (13).

RESULTS

CD Spectroscopy

Changes in the secondary and tertiary structure of DNA upon complexation with different cationic polymers (Fig. 1) were studied by CD spectroscopy. Typical spectra ($\Delta\varepsilon$ versus λ) for naked DNA and DNA in the presence of different amounts

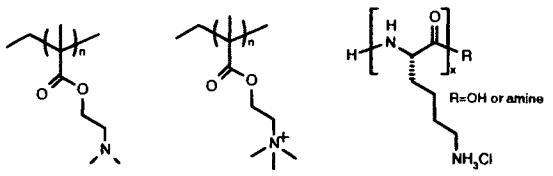

Fig. 1. Structures of the polymers used in this study.

of HMW p(DMAEMA) are shown in Fig. 2. The CD spectrum of naked DNA contains a positive peak with a maximum at 274 nm and a negative peak with a minimum at 246 nm (see Fig. 2), which is typical for B-form DNA (14–15). With increasing p(DMAEMA)/plasmid ratios the λ_{max} for both the positive and negative peak increased and reached a maximum at 0.75-1/1 (w/w) ratio. Simultaneously, a decrease in $\Delta\varepsilon$ of the positive peak and a dramatic increase in $\Delta\varepsilon$ of the negative peak were recorded. These changes in the positive $\Delta\varepsilon$ value can be explained by a transition of the secondary DNA structure from the B to the C type, as a result of dehydration and/or neutralization of the phosphate groups (16). The changes in the negative peak reveal most likely the presence of (parts of) DNA helices oriented parallel to each other, so-called Ψ-DNA (16–17).

Polymers other than p(DMAEMA) also induced a change in the DNA structure from the B to the C form. No differences in the extent of B to C transitions were observed. All polymers studied also induced Ψ-DNA formation. However, small differences were found here: less Ψ-DNA formation was observed in p(TMAEMA) polyplexes than in others (Fig. 3a–b).

Figure 4 shows $\Delta\lambda$ for DNA complexed with p(DMAEMA) (Fig. 4a) and p(TMAEMA) (Fig. 4b) at a polymer/DNA ratio of 3/1 (w/w) as a function of the NaCl concentration of the buffer solution. At increasing NaCl concentration (up to 0.5 M) a strong shift to higher wavelengths was observed for both the maximum and minimum peak. At a NaCl concentration of 0.75 M or higher, the wavelength of both peaks was similar to the one of naked DNA. A control experiment showed that the CD spectrum of naked DNA was independent of the NaCl concentration (0-1.5 M). It is therefore concluded that NaCl at concentrations below 0.5 M enhances the B to C

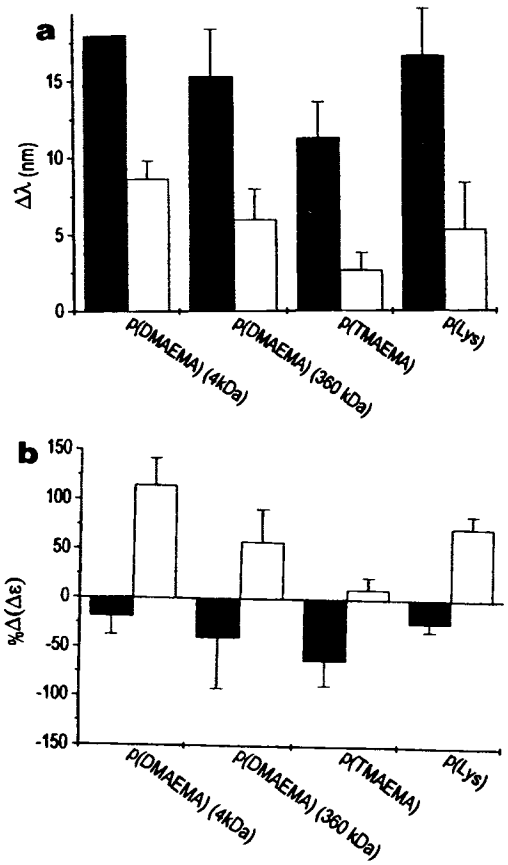

Fig. 3. Changes in the CD spectrum of plasmid-DNA (13.2 μg/mL) by addition of increasing amounts of polymer. (a) $\Delta\lambda$ gives a shift in wavelength for the positive peak maximum (closed bars) and negative peak minimum (open bars). (b) $\Delta(\Delta\varepsilon)$ is the difference (before and after addition of polymer) in the $\Delta\varepsilon$ in the maximum (closed bars) and minimum (open bars). The polymer/plasmid ratio used was 1/1 (w/w). The results are shown as mean values (± SD) of 3 measurements.

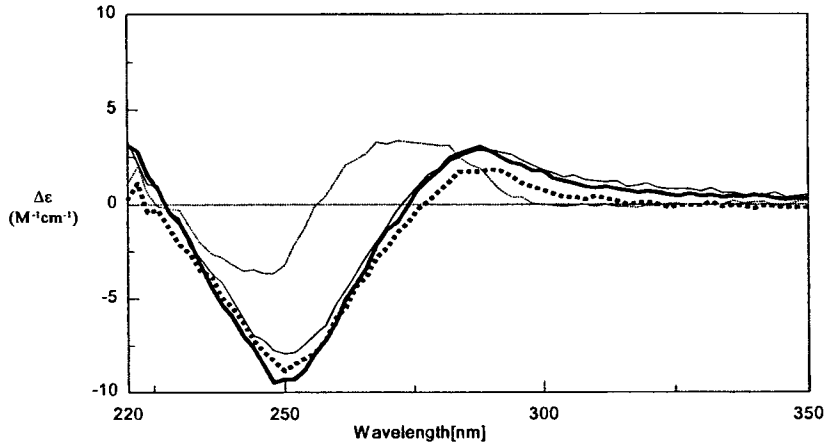

Fig. 2. Typical CD spectra of free plasmid DNA (---) and p(DMAEMA)-plasmid DNA complex at a 0.75/1 (- - -), 1/1 (▬) and 3/1 (——) (w/w) ratio. DNA concentration was 13.2 μg/mL.

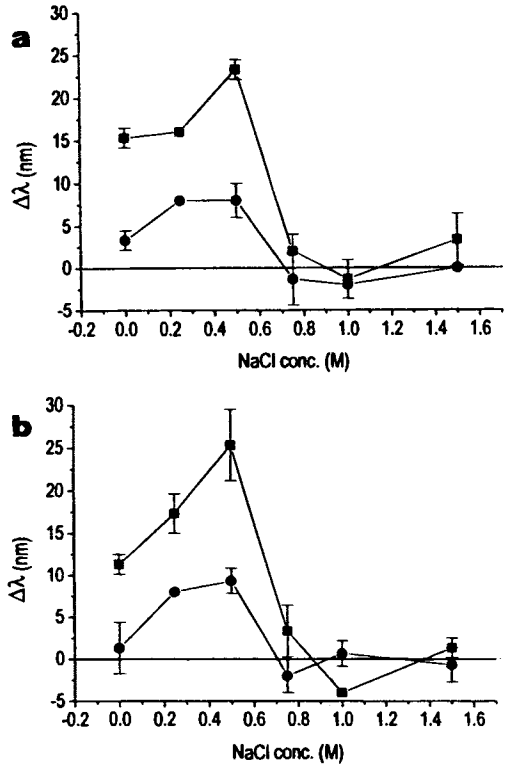

Fig. 4. Changes in the CD spectra of DNA (13.2 μg/mL) complexed with p(DMAEMA) (a) or p(TMAEMA) (b) by addition of increasing amounts of NaCl (final concentrations ranging from 0.25–1.5 M). Δλ is the wavelength shift for the positive peak maximum (■) and negative peak minimum (●). The polymer/plasmid ratio was 3/1 (w/w). The results are shown as mean values (± SD) of 3 measurements.

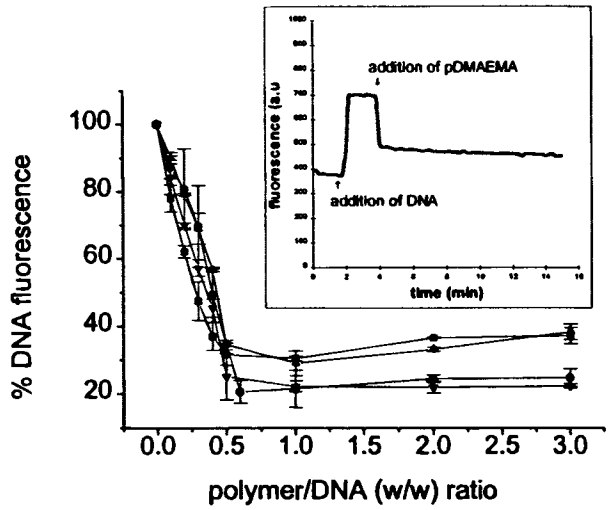

Fig. 5. Fluorescence of an acridine orange (2 μg/mL) solution at a fixed DNA concentration (10 μg/mL) and a varying polymer/DNA (w/w) ratio. The symbols correspond to: (■) HMW p(DMAEMA), (●) p(TMAEMA), (▲) LMW p(DMAEMA) and (▼) p(Lys). The results are shown as mean values (± SD) of 3 measurements. Inset: Typical example of the fluorescence increase of acridine orange upon addition of DNA after 2 min. Upon addition of p(DMAEMA) after 4 min, the signal decreases.

conversion of DNA in the polyplexes, suggesting further dehydration of the DNA. At concentrations above 0.75 M NaCl dissociation of the polyplexes occurs. The conformational change of DNA was also reversed when polyplexes were incubated in the presence of the anionic polymer dextran sulfate at a concentration of 100 μg/mL (data not shown). P(Asp) was also used to dissociate polyplexes. However, this polyaminoacid possesses its own CD spectrum in the range of interest, which interferes with the DNA spectrum.

Fluorescence Spectroscopy

When acridine orange intercalated in DNA, an enhancement of the fluorescence of this dye was observed, probably caused by the disability of oxygen to quench the fluorescence of this probe under these conditions. Addition of a cationic polymer (e.g., p(DMAEMA)) to this mixture instantaneously decreased the fluorescence intensity (Fig. 5, inset). The results are presented in Fig. 5 for all the polymers used in the study. It is shown that for polymer/DNA ratios ≤ 0.5–0.6 (w/w) the decrease in fluorescence was a linear function of the polymer/DNA (w/w) ratio. Above these ratios the fluorescence intensity was constant: ca. 20 to 30% of the initial intensity (Fig. 5). The order found for the plateau values was: p(Lys) ≈ p(TMAEMA) < p(DMAEMA) with different molecular weights. Partial recovery of the fluorescence occurred after the addition of dextran sulfate. The order of the recovery was: HMW p(DMAEMA) ≈ p(TMAEMA) ≈ p(Lys) < LMW p(DMAEMA) (data not shown). The differences found are statistically significant (t-test, $p < 0.05$) in all cases. However, the fluorescence could not be totally recovered and the recovery decreased with longer incubation times of the polyplexes. Moreover, p(Asp) induced a higher recovery of fluorescence than dextran sulfate (Fig. 6).

Agarose Gel Electrophoresis

Polyplexes of p(DMAEMA), p(TMAEMA) or p(Lys) at different ratios were studied by agarose gel electrophoresis. With increasing polymer/DNA ratios, the amount of free DNA

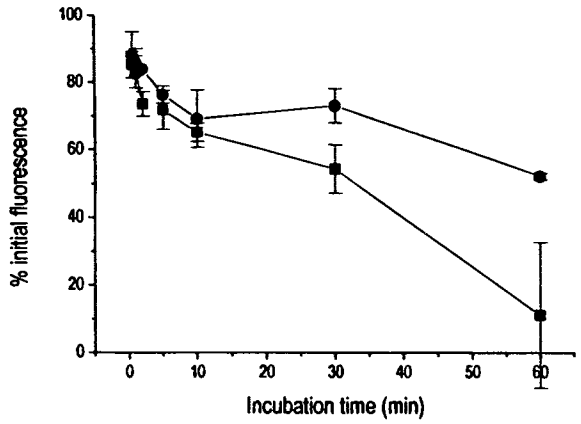

Fig. 6. Fluorescence of acridine orange in the presence of p(DMAEMA)/DNA complexes as a function of complexation time and after the addition of dextran sulfate (100 μg/mL) (■) or p(Asp) (100 μg/mL) (●). The polymer/DNA ratio was 3/1 (w/w). Results are shown as mean values (± SD) of 3 measurements.

(open circular and supercoiled) decreased. Above a 0.4/1 (w/w) ratio no free DNA could be detected (Fig. 7a); DNA remained in the slot. Interestingly, it was not possible to visualize DNA remaining at the application site for p(TMAEMA)/DNA complexes. Fig. 7b shows the electrophoretic pattern of DNA after incubation of polyplexes with an excess of p(Asp). The amount of dissociated DNA observed in the agarose gel was higher with p(Asp) as dissociation agent when compared with dextran sulfate (not shown). Polyplexes composed of DNA and HMW p(DMAEMA), LMW p(DMAEMA) or p(Lys) showed naked DNA at every ratio studied. Interestingly, DNA did not dissociate from p (TMAEMA)-containing polyplexes treated with p(Asp). When analyzing polyplexes composed of DNA and p(Lys) or LMW p(DMAEMA), free DNA was observed and no DNA was detected in the slot, which suggests complete dissociation. Electrophoresis of polyplexes consisting of DNA and p(TMAEMA) or HMW p(DMAEMA) only showed free DNA at low polymer/DNA ratios (0.4–0.2 (w/w)). At higher polymer/DNA ratio (0.4–0.8 (w/w)), no free DNA was observed for p(TMAEMA) and only partial dissociation was observed for HMW p(DMAEMA). At polymer/DNA ratio of 3/1 (w/w), for p(TMAEMA) only partial dissociation was found, whereas for HMW p(DMAEMA)the dissociation was complete.

Nuclease Resistance of p(DMAEMA)/DNA Complexes

Polyplexes of different compositions protected the plasmid against enzymatic digestion with DNase I, as showed by the absence of an increase in the UV absorption after addition of the enzyme (not shown). This has been reported for other polyplexes as well. We studied the resistance of DNA complexed with different polymers against degradation by DNase I in more detail by studying the DNA topology with agarose gel electrophoresis (Fig. 7c). HMW p(DMAEMA) and p(Lys) protected DNA against degradation at a polymer/DNA ratio of 0.2 (w/w) or higher; the DNA structure was almost completely preserved, although a decrease in the supercoiled/open circular DNA ratio was observed. LMW p(DMAEMA) protected DNA at polymer/DNA ratios ≥ 0.4, but in all cases only open circular DNA could be observed. Since p(TMAEMA)-based polyplexes could not be dissociated by p(Asp), the integrity of the plasmid could not be evaluated.

Transfection Experiments and Influence on Cell Viability

The influence of the polymer used as carrier for DNA on the cell viability and transfection efficiency was evaluated in a tissue culture system in the presence of serum. Table 1 shows the relative in vitro transfection efficiency and relative cell viability of the different polyplexes used. For all cationic polymers a bell-shaped dependence of transfection efficiency on the polymer/DNA ratio was observed. As found before (6) HMW p(DMAEMA) at a 3/1-6/1 polymer/DNA (w/w) ratio showed the highest transfection efficiency. In agreement with previous observations, p(DMAEMA) with a low molecular weight had a poor transfection efficiency and a very low cytotoxicity. This might be caused by the inability of this polymer to condense DNA and form small nanoparticles (2).

As previously (6) observed, p(TMAEMA) and p(Lys) were very poor transfectants. However, their cytotoxicity is different: cell viability for p(TMAEMA) was over 80% and over 60%

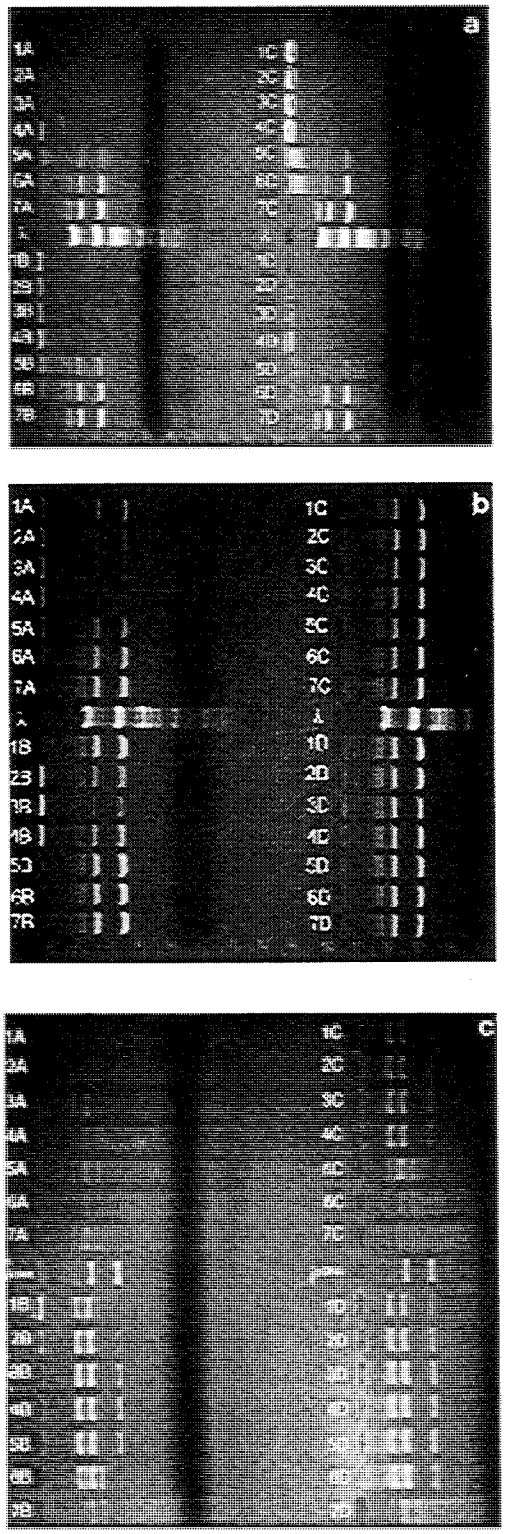

Fig. 7. (a) Agarose gel electrophoresis of p(TDMAEMA)/DNA (A), HMW p(DMAEMA)/DNA (B), LMW p(DMAEMA)/DNA (C) and p(Lys)/DNA (D) complexes in 20 mM Hepes, pH 7.4, at different polymer/DNA (w/w) ratios: 3/1 (1), 1/1 (2), 0.8/1 (3), 0.6/1 (4), 0.4/1 (5), 0.2/1 (6) and 0/1 (7). The marker lambda-Hind III (λ) was used as a reference. (b) Agarose gel electrophoresis of DNA dissociated by p(Asp) from p(TDMAEMA)/DNA (A), HMW p(DMAEMA)/DNA (B), LMW p(DMAEMA)/DNA (C) and p(Lys)/DNA (D) complexes in 20 mM Hepes, pH 7.4. Caption as in Figure 7a. (c) Agarose gel electrophoresis of DNA dissociated from polyplexes after Dnase I treatment. Caption as in figure 7a. Free DNA (non treated) was used as a reference.

Table 1. Relative Transfection Efficiency and Cell Viability for Polymer/DNA Complexes

Polymer	polymer/DNA ratio (w/w)	Relative transfection efficiency (%)	Relative cell viability (%)
p(DMAEMA)	0	2 ± 0	91 ± 7
LMW	1.5	2 ± 0	90 ± 6
	3	2 ± 0	91 ± 4
	6	3 ± 0	93 ± 4
	12	3 ± 0	93 ± 4
p(DMAEMA)	0	2 ± 0	91 ± 7
HMW	1.5	4 ± 0	96 ± 3
	3	46 ± 3	81 ± 1
	6	100 ± 7	47 ± 8
	12	21 ± 5	6 ± 2
p(TMAEMA)	0	2 ± 0	91 ± 7
	1.5	2 ± 0	100 ± 0
	3	2 ± 0	91 ± 2
	6	2 ± 0	80 ± 3
	12	2 ± 0	62 ± 14
poly(lysine)	0	2 ± 0	91 ± 7
	1.5	3 ± 0	91 ± 1
	3	3 ± 0	81 ± 2
	6	3 ± 0	53 ± 4
	12	3 ± 0	0 ± 1

Note: Transfection values are normalized to the maximum number of transfected cells after incubation with HMW p(DMAEMA)/DNA 6/1 (w/w) complexes. The cell viability results were normalized to the result obtained for non-treated cells. The results are shown as mean values (±SD) of 3 experiments.

at polymer/DNA ratios of 6 and 12, respectively, whereas p(Lys) showed a much lower cell viability at these ratios (53% and 0% cell viability at 6/1 and 12/1 (w/w) ratio, respectively). This is in agreement with previous results from studies performed in the absence of serum.

DISCUSSION

CD spectroscopy revealed a conformational change in the secondary structure of DNA (Fig. 2) upon association with different cationic polymers, i.e., a partial transition from the B to the C form, a variation of the B form differing in various helical parameters (18). This transition was previously reported for cationic lipid/DNA complexes, p(Lys)/DNA complexes, and for free DNA in solution with high concentrations of cations (15–16,19). Furthermore, the intensity of the negative CD maximum suggests the formation of so-called-DNA Ψ-DNA upon complexation, as previously found for lipid/DNA and p(Lys)/DNA complexes (16–17). Ψ-DNA is an aggregate of DNA with (part of) the chains oriented close and parallel to each other. These changes in DNA were independent of the type of polymer, although differences were found in the extent of these transitions. The changes were maximal at a polymer/DNA ratio ≥ 0.5/1 (w/w). This indicates that an equimolar amount [0.5/1 (w/w) ≈ 1/1 (mol/mol)] of cationic groups was sufficient to neutralize the DNA phosphates. At pH 7.4 in only 50% of the tertiary amines of p(DMAEMA) is charged (20), whereas the quaternary amines of p(TMAEMA) are permanently charged. However, no difference between the neutralization of DNA phosphates by these two polymers were observed, indicating that protonation of amine groups of p(DMAEMA) was induced by the presence of the negatively charged DNA phosphates.

All polymers induced quenching of the fluorescence of acridine orange intercalating with DNA (Fig. 5), although to different extents. This might indicate different strengths in the interaction between DNA and the different polymers. We found that maximal quenching was dependent on the probe, salt concentration and probe/DNA ratio (data not shown). The results obtained here are very similar to those reported in the literature with other polyplexes or lipoplexes (21–22) and with other probes. It has been speculated that the decrease in the fluorescence is the result of direct quenching of the probe by the polymer and/or a decreased intercalation of the probe into DNA. The emission spectrum of acridine orange is different when it interacts with RNA (23). However, we did not observed any shift in the wavelength of the maximal emission here.

Acridine orange was not able to interact properly with DNA complexed with cationic polymers. The same was found for the enzyme DNase I. Both p(DMAEMA) and p(Lys) provide protection against digestion by DNase I after a reorganization takes place inside the complex (Fig. 7c). This was previously reported for complexes of DNA and p(Lys) or PEG-p(Lys) block (co)polymers (24–25). The protection provided by p(DMAEMA) and p(Lys) is definitely an advantage under physiological conditions when degradation through nuclease attack readily takes place.

Free DNA was observed at a polymer/DNA ratio ≤ 0.4/1 (w/w) by agarose gel electrophoresis (Fig. 7a). We suppose that polymers are randomly distributed over the plasmids upon mixing and that during electrophoresis a reorganization of this distribution takes place, so that part of the DNA will be able to run through the gel.

The dissociation characteristics of polyplexes were studied to obtain information about the strength of the interaction between the cationic polymer and DNA. Dissociation of polyplexes is essential in the transfection process, because only released and intact DNA can be transcribed into RNA. CD spectroscopy experiments showed that the structure of free DNA was recovered by the addition of dextran sulfate or ≥ 0.75 M NaCl to p(DMAEMA)/DNA 3/1 (w/w) complexes (Fig. 4). However, the quenching of acridine fluorescence could only be partially reversed by the addition of dextran sulfate and depended on the time of polyplex formation (Fig. 6). This suggests that in complexes of DNA and p(DMAEMA) or p(TMAEMA), a reorganization is taking place after complexation, leading to polyplexes that are more difficult to dissociate. A maturation process has also been described for cationic liposome/DNA complexes, leading to higher serum-resistance (26) in transfection experiments.

Experiments with agarose gel electrophoresis also indicated that polyplexes could not be completely dissociated with dextran sulfate (data not shown). However, when p(Asp) was used to dissociate the complexes prior to electrophoresis, free DNA was observed at higher polymer/DNA ratios (Fig. 7b) and the DNA had a topology similar to that of the control (naked DNA). The dissociation of DNA from complexes with p(DMAEMA) or p(Lys) was almost complete. Interestingly, the amount of released DNA observed with p(TMAEMA)/DNA complexes was lower, indicating that the DNA is more difficult to (totally) dissociate from this polymer. Wink *et al.* (27) found

by surface plasmon resonance higher apparent dissociation rates for the interaction between plasmid DNA and p(DMAEMA) coated on a gold plate than for the interaction between plasmid DNA and p(Lys). Our experiments did not confirm this observation.

The ultimate aim of this study was to provide a characterization method for polymer/DNA complexes that can predict the transfection efficiency of these systems. We are aware of the fact that the methods used to dissociate are artificial. However, in particular the polyanions used in the study may mimic the situation the polyplexes encounter inside cells: the presence of negatively charged biopolymers (proteins, RNA and even DNA). As previously observed, p(TMAEMA), p(Lys) and LMW p(DMAEMA) were very poor transfectants, in contrast to HMW p(DMAEMA) (Table 1). Experiments with CD spectroscopy, fluorescence measurements with acridine orange, and agarose gel electrophoresis did not show major differences in the characteristics of complexed DNA in polyplexes with very different transfection efficiency. A critical step in the transfection process might be the dissociation of polyplexes. Experiments with agarose gel electrophoresis provide interesting information about the capability of polyplexes to dissociate. We showed that DNA was easier to dissociate from p(DMAEMA) than from p(TMAEMA) and, indeed, the transfection efficiency is better for p(DMAEMA) than for p(TMAEMA). This is in accordance with the hypothesis in a recent paper of our group (6) that defines the endosomal escape and the dissociation of DNA from the polymer as the critical steps to be studied for improving the transfection efficiency of these systems. However, other polymer characteristics will certainly play a role as well. First, LMW p(DMAEMA) was inefficient as transfectant, although DNA can be easily dissociated from it. This is probably caused by the inability of this polymer to condense DNA (2, 5). HMW p(DMAEMA) condenses DNA to form stable nanoparticles and DNA could be totally dissociated from it. This polymer showed the best transfection efficiency in vitro of all the polymers studied. Second, p(Lys) and DNA formed stable nanoparticles and could be dissociated (see Fig 7b), but due to the high cytotoxicity of p(Lys) and probably to its inability to act as a proton sponge, helping to escape the endosome, it is a poor transfectant (2,20).

In conclusion, we characterized the association and dissociation properties of polymer/DNA complexes in detail. Insight into these charcteristics may turn out to be very helpful in further rationalizing the process of optimizing the performance of non-viral gene delivery systems.

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