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# Poly(diallyldimethylammonium chlorides) and their *N*-methyl-*N*-vinylacetamide copolymer-based DNA-polyplexes: role of molecular weight and charge density in complex formation, stability, and in vitro activity

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**Abstract**

Poly(diallyldimethylammonium chlorides) (pDADMAC) of different molecular weights (5–244 kDa) and DADMAC/*N*-methyl-*N*-vinylacetamide (NMVA) copolymers (coDADMAC) with different composition (24–75 mol%) and therefore varying cationic charge densities were used to investigate the relationship between polymer structure, polyplex formation and stability, as well as their biological activity. All polymers interacted electrostatically with DNA to form polyplexes as detected by electrophoresis. Complexation and condensation of DNA by the polycations as well as protection of DNA against mechanical and enzymatic degradation were found to increase with higher molecular weights and charge densities of the polymers as well as increasing charge ratios of the complexes. Static and dynamic light scattering revealed for all DNA/polymer complexes sphere-like structures of about 100–150 nm forming more compact structures with increasing charge ratios which were stable over 24 h. The in vitro cytotoxicity of the free polymers determined by MTT-assay was directly correlated to molecular weight and charge density of the polycations which was also confirmed for polymer/DNA complexes quantifying the membrane toxic effects by LDH-release. The transfection efficiency of the complexes was low independent from different charge ratios, presence or absence of serum and lysosomotropic agents. In conclusion, the DADMACs are an interesting tool to study structure-function-relationships due to the specific adjustment of molecular weight as well as number and density of charges.


*Keywords:* Gene delivery; Poly(diallyldimethylammonium chloride); DNA interaction; Stability; In vitro cytotoxicity; Structure-function-relationship

## 1. Introduction

Polymeric macromolecules with different structures, linear or branched, or block and grafted copolymers with varying numbers and p$K_a$ of the protonable amines in backbone or side chain have been described to form polyelectrolyte complexes by self-assembly with DNA or RNA as nonviral gene delivery systems (De Smedt et al., 2000). These polymers differed concerning their cytotoxicities and efficiencies in transferring genetic information, rising the question for structure-function-relationships and inducing

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many attempts to understand and control the process of nonviral gene delivery.

Poly(diallyldimethylammonium chlorides) (pDADMACs) are a class of cationic polymers which were widely used for different technical applications such as flocculant in water treatment, for the separation of oil–water-emulsions, as ionic retention aid and precipitant in paper industry, as well as for the microencapsulation of enzymes (Jaeger et al., 1989). However, pDADMAC has not been described so far for medical and biological applications. Although the cationic charge of this polymer suggests a potential as gene delivery system, no information about binding, protection and transfection of DNA or biocompatibility has been reported so far.

pDADMAC is a water soluble, linear homo-polymer with quarternary ammonium groups on the rings included in the backbone of the polymer chain. Therefore, the polymer tends to be quite stiff, having a longer persistence length than, for instance, the polyamines used as gene delivery agents and a more extended conformation in solution (Hubbe, 2003). Furthermore, the pDADMACs differ from polyamines such as polyethylenimine and dendrimers due to their permanent cationic charge independent from the pH of the surrounding medium which offers an interesting possibility to study the influence of the number of cationic charges and the charge density on the properties of gene delivery systems (Dautzenberg and Jaeger, 2002).

In the present study, a series of DADMAC homopolymers with different molecular weights and a second series of DADMAC copolymers with noncharged *N*-methyl-*N*-vinylacetamide (NMVA) with different cationic charge densities were used to systematically investigate the influence of polymer structure on the interaction with DNA, the stability of the polymer/DNA complexes against enzymatic and mechanical treatment as well as in vitro cytotoxicity and transfection efficiency. Four homo-polymers of DADMAC (pDADMAC) with the molecular weights 5, 45, 135 and 244 kDa were used. Except from the 5 kDa product, the $M_w/M_n$ ratio of the pDADMACs was below 2. In 0.5 M NaCl solutions the persistence length was found to be 2.5 nm (Dautzenberg et al., 1998) proving a partially flexible chain structure. On the other hand, three copolymers made of DADMAC and NMVA (coDADMAC) with similar molecular weights in the range of 92–107 kDa, comparable $M_w/M_n$ ratios about 1.45, and different cationic charge densities due to varying mol% DADMAC content (24, 53 and 75 mol%) were investigated. The structure of the copolymers consisted of two different parts in each repeating unit: The aminic residue of the DADMAC monomer which is permanently cationized and the amidic part, contributed by the NMVA comonomer which is not ionized under physiological conditions.

## 2. Materials and methods

### 2.1. Polymers

All polymers were a kind gift of Dr. Werner Jaeger, Frauenhofer Institute of Applied Polymer Research, Golm, Germany. The pDADMACs were prepared by radical polymerization in aqueous solution using 2,2′-azo-di-2-methylpropanamidine dihydrochloride as initiator according to Dautzenberg et al. (1998). Experimental conditions for the synthesis of polymers with different molecular weight were derived from kinetic data of the polymerization process. Monomer concentration was varied between 1.0 and 4.0 mol/L, initiator concentration between 2E-2 and 2E-3 mol/L, and the reaction temperature between 30 and 50 °C. Diallyldimethylammonium chloride (60 wt.% aqueous solution, Aldrich, Taufkirchen, Germany) and 2,2′-azo-di-2-methylpropanamidine dihydrochloride (V 50, Wako Chemicals GmbH, Neuss, Germany) were used without further purification. The polymerization was stopped at low conversion (5–10%) by cooling and diluting. The polymers were purified by ultrafiltration (cut off: 10 kDa), isolated by freeze drying, and characterized as described in Dautzenberg et al., 1998. The copolymers poly[(diallyldimethylammonium chloride)-co-(*N*-methyl-*N*-vinylacetamide)] were synthesized and characterized as described in Ruppelt et al. (1997) and Brand and Dautzenberg (1997).

### 2.2. DNA

The 6.985 kb plasmid pCMV-nlacZ encoding β-galactosidase under the control of the human cytomegalovirus promotor was a generous gift of Aventis, Frankfurt a.M., Germany. The pGL3 control plasmid (Promega, Mannheim, Germany) coding for

the photinus pyralis luciferase gene was amplified using JM-109 competent cells (Promega), isolated and purified using the QIAfilter Mega Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Concentration and purity of the pDNA were determined by 260/280 UV absorbance ratio and integrity by agarose gel electrophoresis. Herring testes DNA sodium salt (Sigma, Deisenhofen, Germany) was purified by ultrafiltration to remove low molecular weight impurities (cut off: 300 kDa). This type of DNA was used as a model for double stranded DNA species in DNA-binding, light scattering experiments, and in vitro cytotoxicity testing where no function of the DNA and/or due to the type of technical equipment high amounts of DNA were necessary.

### 2.3. Preparation of polymer/DNA complexes

Nine milligrams polymer were diluted into 8 mL of bidistilled water, adjusted to pH 7.4 with 1 N hydrochloric acid, brought to 10 mL and sterile filtered (0.2 μm). Ten micrograms DNA and the desired amount of polymer were each diluted into 250 μL with 150 mM NaCl pH 7.4 and vortexed. After 10 min at room temperature, the polymer solution was added to the DNA solution and the resulting mixture was vortexed again. The complexes were allowed to equilibrate 10 min at room temperature prior to experimentation. The complex composition was calculated on the basis of positive charge equivalents of the polycation per negative charge equivalents of the DNA and expressed as charge ratio according to Felgner et al. (1997).

### 2.4. Agarose gel electrophoresis for determination of DNA binding and complex stability

#### 2.4.1. Gel electrophoresis

Fifty microliters aliquots of the herring testes DNA/polycation complex solution containing 10 μg DNA were loaded onto an ethidium bromide-containing 1% agarose gel (Roth GmbH, Karlsruhe, Germany) and were electrophoresed (Blue Marine 200, Serva, Heidelberg, Germany) at 80 V (LKB 2197 Power Supply, Pharmacia, Germany) for 2 h in TAE buffer (40 mM Tris–base, 1% (v/v) acetic acid, 1 mM EDTA). Gels were photographed (Video Copy Processor P67E, Mitsubishi) under UV transillumination (TC-254A Transilluminator, 254 nm).

#### 2.4.2. Complex stability

For investigation of the mechanical stability CMV-nlacZ/polymer complexes were ultrasonicated (Branson Sonifier 250, Branson, Germany, 70% duty cycle, 20–25% output) for 15, 30 and 60 s. To avoid overheating samples were placed on ice. The enzymatic resistance was characterized by incubation of CMV-nlacZ/polymer complexes with 5 U nuclease/μg DNA (DNase I, 5 mg/ml in 150 mM NaCl and 20 mM $MgCl_2$, pH 7.4, Boehringer Mannheim, Mannheim, Germany) at 37 °C. The reaction was stopped by inactivation of the enzyme at 70 °C for 30 min. After mechanical or enzymatic treatment, DNA was liberated by incubation of the complexes with dextrane sulphate solution (Mw 5000 Da, Sigma) for 10 min and separated by electrophoresis (80 V, 2 h) as described above.

### 2.5. Investigation of complex structure by light scattering

#### 2.5.1. Instrumental

Static light scattering measurements were carried out with a Sofica 42000 instrument (Wippler and Scheibling, Strasbourg, France) and also with the SIMULTAN (ALV, Langen, Germany). The Sofica instrument was equipped with an intensity stabilized 1 mW He–Ne-laser (Spectra Physics) as light source and a PC for data recording. An ALV goniometer with an ALV-5000 digital time correlator and a 400 mW ($\lambda = 532$ nm) laser DPSS 532-10 (Coherent, USA) was used for simultaneous static and dynamic measurements. Three runs per 30 s were carried out to calculate the average scattering intensities at each angle.

#### 2.5.2. Static light scattering

For the quantitative analysis of the light scattering data the concentrations $c_{PEC}(X)$ of the polyelectrolyte complexes (PEC) and their refractive index increments $\nu_{PEC}(X)$ must be known in dependence on the molar charge ratio $X$ of the anionic to cationic groups or vice versa. In the case of polyanion solutions as starting one $c_{PEC}(X)$ is given by the expression:

$$c_{PEC}(X) = c_A \frac{V_A}{V_A + V_C} \left( \frac{m_A f(X) + m_C}{M_A} \right) X \qquad (1)$$

where $c_A$ is the mass concentration of the polyanion in the starting solution, $V_A$, $V_C$ the volume of the starting solution and the added volume of the polycation solution, respectively, $m_A$, $m_C$ the molar masses per charged unit of the polyanion and polycation without the counterions, respectively, $M_A$ the corresponding molar mass with the counterion and $f(X)$ is the stoichiometric factor. Assuming a 1:1 stoichiometry this expression is valid up to $X = 1$, after that $X = 1$ has to be used.

For the refractive index increment one obtains:

$$\nu_{PEC}(X) = (\nu_A m_A + \nu_X m_C)/(m_A + m_C) = 0.187 \text{ ml/g} \quad (2)$$

where $\nu_A$ (=0.185 ml/g) and $\nu_C$ (=0.19 ml/g) are the increments of the components.

The static light scattering data were analyzed by a Zimm plot (Zimm, 1948):

$$\frac{K c}{R(q)} = \frac{1}{M_w} + \frac{R_G^2 q^2}{3M_w} \quad (3)$$

where $R(q)$ is the Rayleigh ratio of the scattering intensity, $K$ a contrast factor containing the optical parameters, $c$ the complex concentration, $M_w$ the weight average of the molar mass of the complex particles, and $R_G$ is their radius of gyration (calculated from the z-average of the square). The concentration dependence was neglected, what seems to be justified because of the low concentrations of the PEC solutions ($\sim 10^{-5}$ g/mL). Extrapolation to zero scattering angle was carried out by quadratic fits of the scattering curves.

### 2.5.3. Dynamic light scattering

The correlation functions were analyzed by second order cumulant fits, providing the z-average of the diffusion coefficient after extrapolation to zero scattering angle (Chu, 1991). From that the hydrodynamic radius $R_H$ was calculated by the Einstein–Stokes formula $R_H = kT/6\pi\eta D$.

The structure sensitive parameter $R_G/R_H$ gives an information about the structure type and has a value of 0.775 for spheres, but values well above 2 for elongated structures.

### 2.5.4. Preparation of complexes for light scattering experiments

All complexes were prepared according to Section 2.3. with minor modifications in 0.1 M NaCl pH 7.4. To 5 mL of a herring testes DNA solution of a mass concentration $c_{DNA} = 3.15 \times 10^{-5}$ g/mL ($\sim 9.4 \times 10^{-5}$ mol/L of phosphate groups, $m_e = 335$) 5 mL of the polycation solution of an appropriate concentration (according to the desired charge ratio 0.75, 1, 2 and 5) were rapidly added under stirring. Both solutions were filtered through a membrane filter of 0.8 μm pore size into the scattering cell of the Sofica instrument to keep the solutions dustfree.

## 2.6. Biological assays

### 2.6.1. In vitro cytotoxicity testing

L929 mouse fibroblasts (DSMZ, Braunschweig, Germany) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 10% fetal bovine serum (Gibco) and 2 mM glutamine at 37 °C, 10% $CO_2$ and 95% relative humidity. The MTT protocol used in this study was described previously (Fischer et al., 2003; Mosmann, 1983) using free, non-complexed polymers. The extracellular presence of the cytosolic enzyme LDH (Fischer et al., 2003) as evidence for cell membrane damage, was quantified using a commercial kit (DG 1340-K, Sigma). Briefly, L929 fibroblasts (250,000 cells/well) precultured on six-well plates for 48 h, were incubated with 2 mL/well herring testes DNA/polymer complex solution for 4 h. Control experiments performed with 0.1% (w/v) Triton X-100 were set as 100% toxicity. The LDH amounts determined were related to the Triton X-100 value and expressed as percent release.

### 2.6.2. Transfection efficiency

Transfection experiments were performed in triplicate as described earlier (Kunath et al., 2003). Briefly, cells were seeded in 12-well plates at a density of 50,000 cells/well. Complexes were formed as described above from 12 μg pGL3 plasmid for one triplicate experiment and the appropriate amount of polymer in 300 μL 150 mM NaCl each. Two hundred microliters of complex solution were added to each well after 10 min and were incubated in the absence or presence of 10% FCS or 10 mM chloroquine for

4 h. Afterwards medium was changed again and the cells were incubated for further 44 h. Luciferase expression was then determined with luciferase assay reagent (Promega).

## 3. Results and discussion

In the present study, we examined the properties of polyelectrolyte complexes formed by self-assembly of DNA with a series of DADMAC homo- and copolymers of varying molecular weight and cationic charge density to investigate the structural impact on physicochemical and biological properties of the systems.

### *3.1. Determination of DNA binding by agarose gel electrophoresis*

Gel electrophoresis (Fig. 1) revealed a polymer structure and charge ratio-dependent selfassembly of the polycations with DNA. To a constant amount of herring testes DNA increasing concentrations of the polymers were added. Free DNA was characterized by a fluorescent smear due to the broad distribution of its molecular weight. As the polymer concentration increased, the amount of DNA migrating into the gel with unchanged electrophoretic mobility decreased. At charge ratios <1 free DNA as well as DNA with different complexation states were detectable, a disproportionation which was also described by others (Jones et al., 2000): one fraction with an intermediate migration suggestive of partially complexed DNA and a second fraction showing retardation of the DNA at the origin indicating that the complexes were larger in size and/or less negatively charged than free DNA.

Whereas total retardation of the DNA was observed at charge ratio 0.84 for the 244 and 135 kDa polymer, the 45 kDa and the 5 kDa pDADMACs immobilized the DNA completely only at higher charge ratios (45 kDa: 0.93, 5 kDa: 1.03). Complete inaccessibility of the complexed DNA to ethidium bromide due to exclusion of the intercalant dye which was interpreted as a reflection of DNA condensation could be observed at charge ratios higher than 3 for all homo-polymers (data not shown). The DNA binding characteristics of the coDADMACs compared to the 135 kDa pDADMAC revealed that a reduction of the cationic density decreased the complexation capacity of the polymers. The almost complete complexation of the DNA by the 24 mol% coDADMAC even at charge ratio 0.56 seemed to be somewhat surprising. However, after addition of the polycation to the DNA a macroscopically visible, randomly aggregated, sedimenting system was formed which was too large to migrate into the gel. Complete ethidium bromide exclusion could not be observed for the three copolymers up to charge ratio 15 (data not shown).

These results indicated that the efficiency of DNA compaction based on electrostatic interactions between the complex components was increased with higher molecular weights and cationic charge densities of the polymers as well as higher charge ratios of the complexes. According observations were reported for quarternized poly(4-vinylpyridine)/DNA complexes showing a less efficient quenching of ethidium bromide with shorter chain lengths and lower degrees of quarternization (Izumrudov et al., 1999). Poly(amido amines) (Jones et al., 2000) and oligocationic peptides (Plank et al., 1999) also demonstrated an increase in DNA condensation with higher cationic charge densities of the polymers. The ability of polyethylenimines to condense DNA and to produce stable, non-aggregating systems increased with higher molecular weights (Kunath et al., 2003). The influence of the charge ratio of the complexes on DNA compaction has been confirmed by others (Ogris et al., 1998). The weak binding of DNA by DADMACs with small molecular sizes and low charge densities may result from a reversible binding of the polycationic molecules to DNA and therefore, rearrangements destabilizing the complexes and favouring aggregation (Tang and Szoka, 1997; Wolfert et al., 1999).

### *3.2. Investigation of complex structure by light scattering*

With regard to compaction of the complexes, all systems behave in a similar way as demonstrated by the scattering curves of the complex DNA/5 kDa pDADMAC in Fig. 2. The static light scattering curves (Fig. 2A) revealed that up to the charge ratio 2 no significant changes of the particle masses occured (nearly the same section on the ordinate), but the slopes of the curves changed drastically. Only for the charge ratio 5 a strong increase of the particle masses could be observed. The decreasing slopes with rising

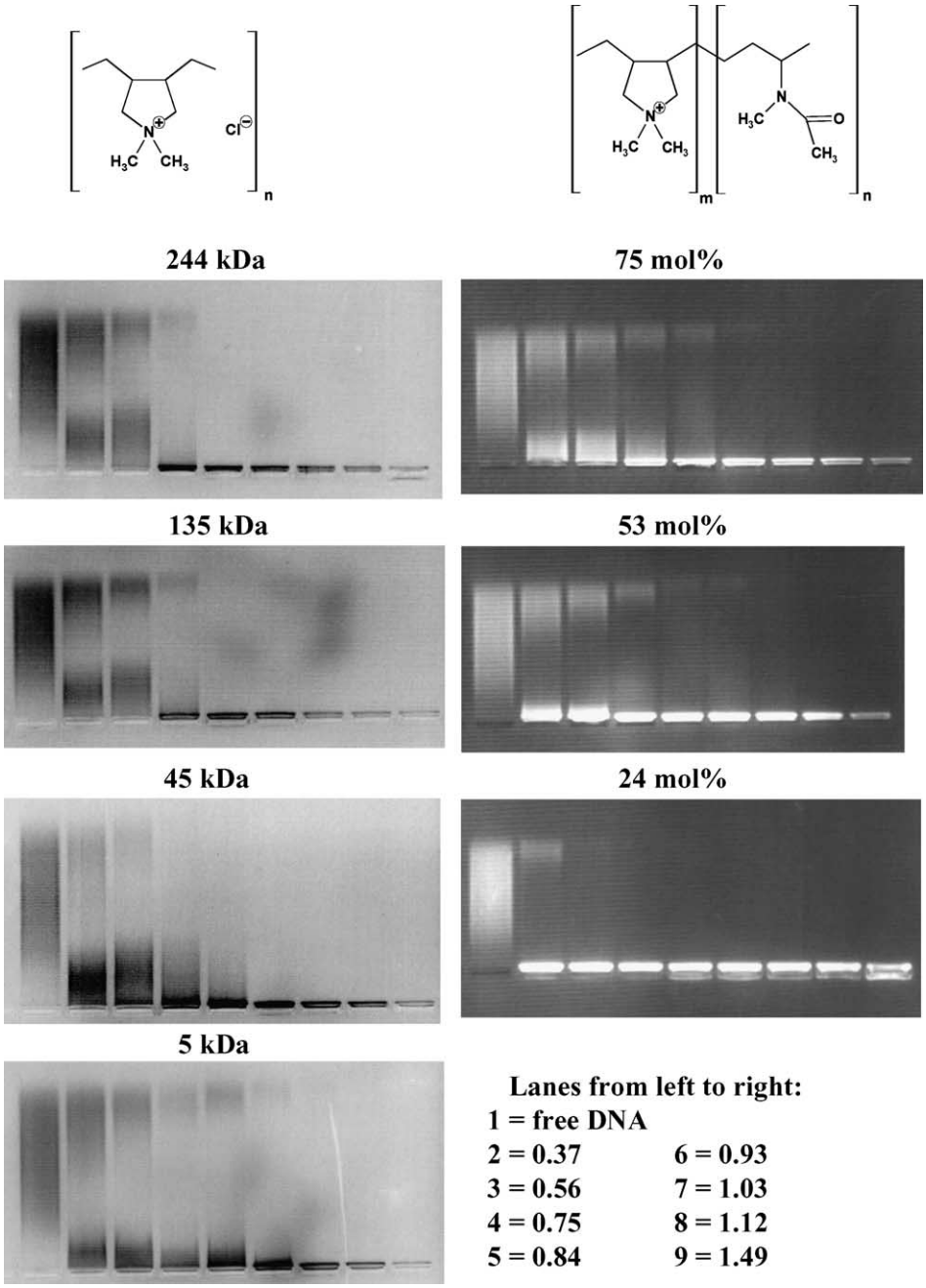


Fig. 1. Analysis of polymer/DNA complex formation. Herring sperm DNA was complexed with the DADMAC polymers at different charge ratios as indicated. Retardation of the DNA by polymers and exclusion of ethidium bromide as indicator for condensation were analyzed by agarose gel electrophoresis.

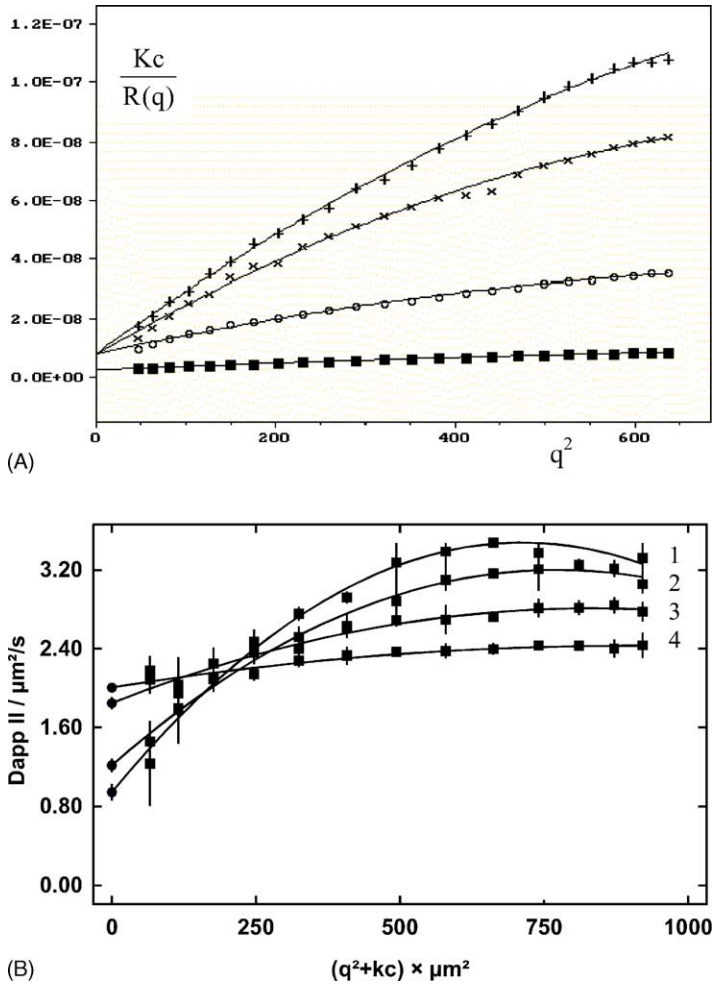


Fig. 2. (A) Zimm plot of the scattering curves of the DNA complexes with 5 kDa pDADMAC in relation to the charge ratio *X*: 0.75 (+), 1 (×), 2 (○), 5 (■). (B) Angular dependence of the diffusion coefficients of the DNA complexes with 5 kDa pDADMAC in relation to the charge ratio *X*: 0.75 (1), 1 (2), 2 (3), 5 (4).

charge ratios indicated clearly the transition to more compact structures which correlated with the DNA binding characteristics of the polymers observed in the electrophoresis experiments. This picture was also confirmed by the behavior of the diffusion coefficients obtained by dynamic light scattering (Fig. 2B). The values extrapolated to zero scattering angle increased with increasing charge ratio, i.e. the hydrodynamic radii decreased. Additionally, the angular dependence became flatter, reflecting the transition to more compact structures.

The structural parameters obtained by a quantitative data evaluation are collected in Tables 1 and 2. To judge the aggregation with regard to the DNA chains the molar mass of the DNA must be known. From gel electrophoresis a molar mass in the range of a few millions should be expected. The value obtained by static light scattering is much higher and ranges from $4 \times 10^7$ to $8 \times 10^7$ g/mol, depending on the filtration conditions (0.45 or 0.8 μm pore size). Since the filtration through the 0.45 μm filter also lowered the scattering level in the wide-angle range, the removal of

Table 1
Structural parameters of DNA/polycation complexes in relation to the molecular masses of pDADMAC

| Polycation $M_w$ (kDa) | Charge ratio | $M_w$ ($\times 10^{-8}$) (g/mol) | $R_G$ (nm) | $R_H$ (nm) | $R_G/R_H$ | $\rho$ ($\times 10^2$) (g/ml)* |
|---|---|---|---|---|---|---|
| 5 | 0.75 | 1.25 | 300 | 240 | 1.25 | 0.36 |
| | 1 | 1.30 | 198 | 186 | 1.06 | 0.80 |
| | 2 | 1.24 | 127 | 124 | 1.02 | 2.40 |
| | 5 | 3.73 | 109 | 114 | 0.96 | 9.98 |
| 45 | 0.75 | 1.37 | 275 | 176 | 1.56 | 1.00 |
| | 1 | 1.25 | 233 | 161 | 1.45 | 1.19 |
| | 2 | 1.36 | 149 | 129 | 1.16 | 2.51 |
| | 5 | 6.86 | 137 | 128 | 1.07 | 13.0 |
| 135 | 0.75 | 1.19 | 219 | 176 | 1.24 | 0.87 |
| | 1 | 1.11 | 183 | 137 | 1.33 | 1.71 |
| | 2 | 1.69 | 116 | 109 | 1.06 | 5.17 |
| | 5 | 22.1 | 184 | 157 | 1.17 | 22.6 |
| 244 | 0.75 | 0.83 | 227 | 186 | 1.22 | 0.51 |
| | 1 | 0.73 | 176 | 130 | 1.35 | 1.32 |
| | 2 | 0.96 | 128 | 116 | 1.10 | 2.44 |
| | 5 | 4.39 | 98 | 98 | 1.00 | 18.5 |

* Calculated by $\rho = M_w/(4\pi/3)R_H{}^3$.

a remarkable amount of DNA must be feared. Therefore, the larger pore size was used for the complex solutions where no loss of DNA could be observed. Most likely, the DNA exists as a mixture of single chain DNA and a small mass fraction of higher aggregated particles, which falsify the $M_w$ obtained by light scattering.

The comparison of the molar masses of the complexes and the DNA showed that the complexes consisted up to the charge ratio 2 of the original DNA system complexed by the polycations. Only the highest charge ratio led to interchain aggregation. For the pure pDADMAC samples this aggregation was more pronounced for pDADMACs with a molar mass near 100 kDa, for the copolymers it increased with increasing amount of NMVA in the copolymers. Accordingly, poly(methacrylate)-based polymers with high cationic charge densities showed tight, discrete complexes detected by atomic force microscopy, whereas polycations containing 50% or less of the charges formed

Table 2
Structural parameters of DNA/polycation complexes in relation to the composition of the copolymers between DADMAC and NMVA ($M_w = 100 \pm 5$ kDa)

| Polycation | Charge ratio | $M_w$ ($\times 10^{-8}$) (g/mol) | $R_G$ (nm) | $R_H$ (nm) | $R_G/R_H$ | $\rho$ ($\times 10^2$) (g/ml) |
|---|---|---|---|---|---|---|
| Mol% of DADMAC 75 | 0.75 | 0.84 | 177 | 154 | 1.15 | 0.91 |
| | 1 | 1.04 | 173 | 151 | 1.15 | 1.20 |
| | 2 | 2.35 | 176 | 148 | 1.19 | 2.87 |
| | 5 | 6.53 | 118 | 115 | 1.03 | 17.0 |
| Mol% of DADMAC 53 | 0.75 | 0.95 | 162 | 151 | 1.07 | 1.09 |
| | 1 | 1.14 | 128 | 144 | 0.89 | 1.51 |
| | 2 | 2.13 | 113 | 127 | 0.89 | 4.12 |
| | 5 | 41.1 | 187 | 172 | 1.09 | 32.0 |
| Mol% of DADMAC 24 | 0.75 | 1.35 | 172 | 144 | 1.19 | 1.79 |
| | 1 | 1.26 | 107 | 144 | Flocculating systems | 9.40 |
| | 2 | 8.43 | 97 | 116 | | 33.1 |
| | 5 | 282 | 363 | 98 | | 21.5 |

more extended structures with lower stability (Wolfert et al., 1999; Howard et al., 2000). The radii of gyration and also the hydrodynamic radii decreased by about a factor 2 in the studied range of the charge ratio. $R_G/R_H$ was near to 1, suggesting a more sphere-like structure. This is a surprising result being aware of the double stranded structure of the DNA, for which much higher values would be expected. However, already $R_G/R_H$ of the DNA was 1.1. Obviously, this DNA exists in 0.1 N NaCl in a pre-aggregated state with a more sphere-like structure, although the estimation of the structural density ($\rho = 1.8 \times 10^{-3}$ g/mL) indicated a highly swollen state. The structural density $\rho$ of the complexes which was calculated from $M_w$ and $R_H$ using the model of a sphere, was higher than for DNA, but not in the expected manner for a fully complexed DNA. Only a high excess of the polycations led to compact packing up to 30 mass% of the polymers in the complexes. However, this high compaction is connected with a strong tendency to aggregation. For the complexes with the 244 and 135 kDa pDADMAC we checked the stability over time. After 1 day the structural parameters were unchanged (data not shown).

The condensation of the DNA is an important prerequisite for the formation of small complexes. With reduced sizes of polyplexes the uptake of particles into cells as well as their diffusion in the cytoplasm were described to increase (Mahato et al., 2000). All of our polymers fullfilled the requirement to form complexes smaller than 200 nm making endocytosis feasable. At charge ratios >1 all complexes had comparable sizes in the range of 98–137 nm with exception of the 135 kDa pDADMAC with 184 nm. This correlated well with Zelikin et al. (2002), who reported that aliphatic ionenes regardless of their molecular weight and charge density formed complexes with DNA with similar particles sizes of about 100 nm. With exception of the 24 mol% DADMAC, all complexes which were characterized in binding experiments by DNA compaction, showed correspondingly in the light scattering experiments a compacted state and small complex sizes.

### 3.3. Determination of complex stability against mechanical and enzymatic treatment

Compaction of DNA by DADMACs can substantially hinder its accessibility to enzymatic or mechanical degradation by physical or electrostatic barrier (Godbey et al., 2000) and thus increase the stability of DNA. To study the integrity of plasmid DNA after enzymatic or mechanical treatment, the DNA has to be released from the complexes. Based on preliminary experiments we used dextran sulphate for the liberation of DNA, which did not influence the integrity and migration of the plasmid by itself. The displacement was more efficient at low charge ratios as well as for complexes formed by polymers with low molecular weight and low charge density (data not shown) and correlated with the reduced DNA compaction observed in electrophoresis and light scattering experiments. Dissociation of DNA from the complexes is one of the critical steps in the transfection process, since only released and intact DNA can be transcribed into RNA.

CMV-nlacZ/pDADMAC complexes of two different charge ratios, with DNA surplus (0.75) and one with excess polycation (1.85) were chosen for stability testing. The analysis of the free expression vector revealed two fluorescent bands corresponding to the supercoiled and open circular form of the plasmid. The plasmid was completely degraded even after 15 s ultrasonication as demonstrated by the formation of broader lanes of lower molecular weight (Fig. 3A). Whereas after 15 s sonication most of the DNA in complexes with charge ratio 0.75 was completely protected by complexation with all pDADMACs, after 30 and 60 s the degradation of the plasmid to lower molecular weight products increased with a decrease of the polymer's molecular weight. This corresponded well to their lower DNA binding efficiency observed in the preliminary experiments. However, only a small amount of DNA could not withstand the high shear forces with increasing sonication times, since the main fraction appeared as intact bands. Nevertheless, an additional third band was observed in all cases between the bands of the supercoiled and the open form. Similar changes in topology of plasmids were also reported by Gebhart et al. (2002) after enzymatic treatment of plasmid/Pluronic-polyethylenimin complexes which need further investigation. Independent of the molecular weight of the pDADMAC, the DNA of all net cationic complexes (charge ratio 1.85) remained intact after 15, 30 and 60 s sonication as a result of the strong compaction of the DNA observed in the binding experiments. For the coDADMAC-complexes with

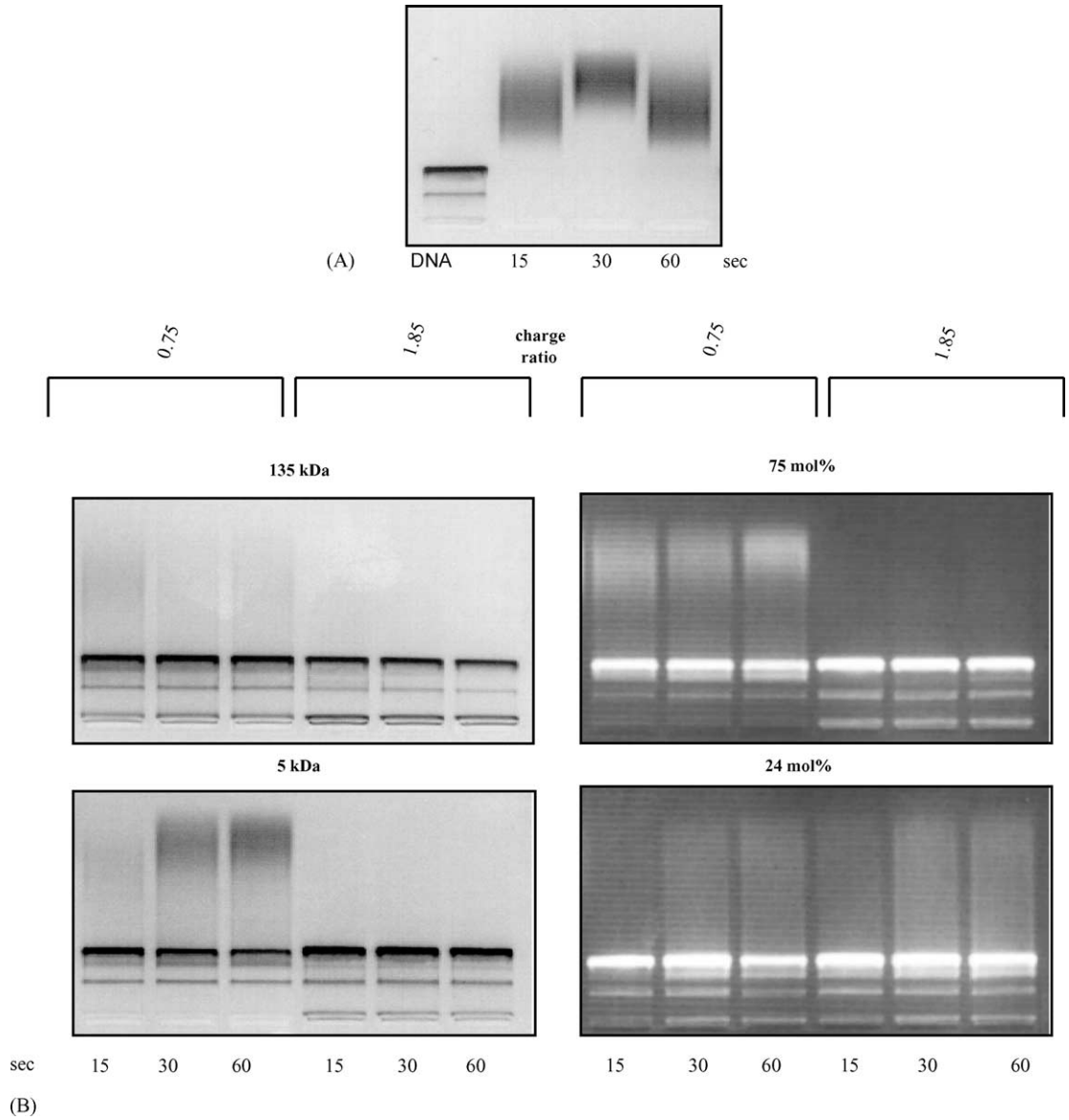


Fig. 3. Effect of ultrasonication on the stability of plasmid DNA. (A) Compared to nontreated CMV-nlacZ plasmid (left lane), plasmid DNA was completely degraded after 15, 30 and 60 s ultrasonication to low molecular weight products. (B) CMV-nlacZ/DADMAC complexes of 135 and 5 kDa pDADMAC and 75 and 25 mol% coDADMAC formed at two different charge ratios (0.75 and 1.85) were sonicated for 15, 30 and 60 s. Complexes were dissociated by dextran sulphate treatment (0.1 mg/μg DNA) and analyzed by agarose gel electrophoresis.

charge ratios 0.75 and 1.85 we found corresponding results with increased stabilization of the DNA with higher charge density of the polycations (Fig. 3B).

Naked plasmid DNA incubated for 5–120 min with the enzyme DNaseI was totally destroyed. Nontreated DNA as well as plasmids incubated for 2 h at 37 °C without enzyme were found to be stable (data not shown). The nuclease treatment of charge ratio 0.75 complexes over 60 min resulted in a complete destruction of the DNA independent of the structure of the

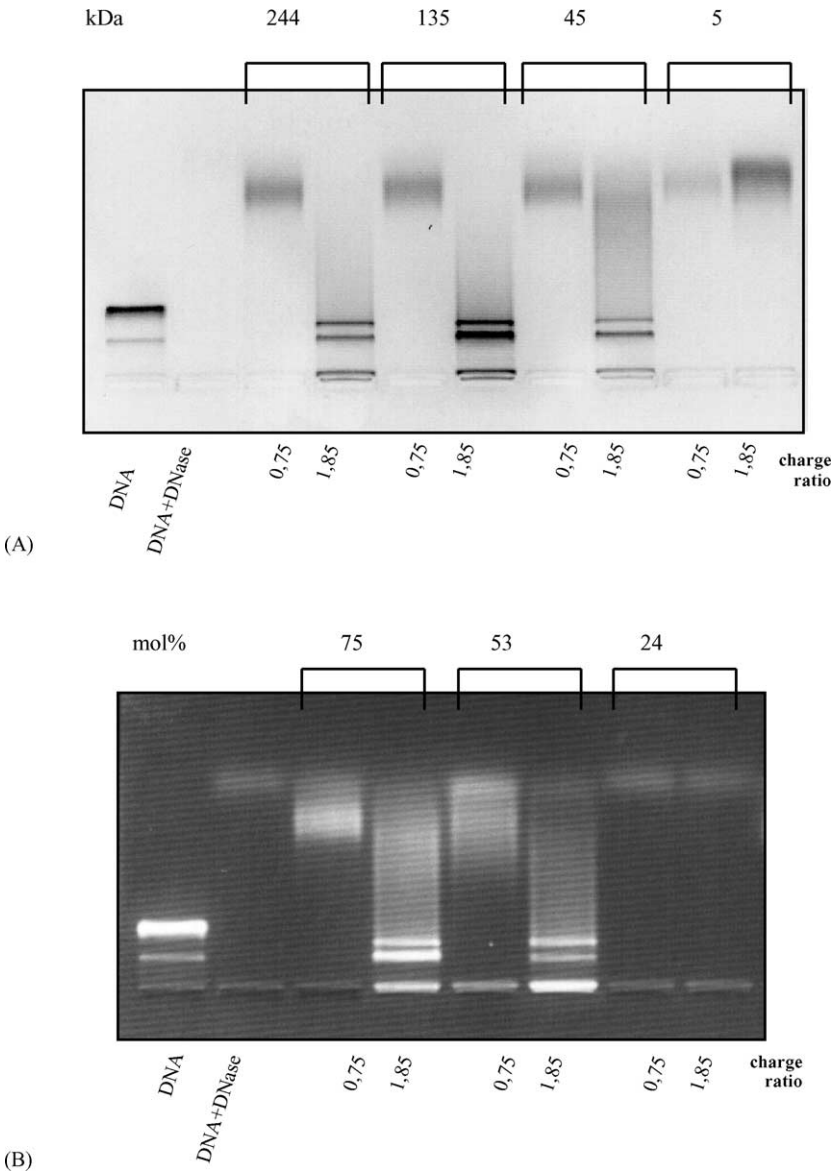


Fig. 4. Stability of plasmid against enzymatic treatment after complexation with (A) pDADMAC and (B) coDADMAC polymers. CMV-nlacZ/polymer complexes with charge ratio 0.75 and 1.85 were incubated with five U DNase I/μg DNA for 60 min at 37 °C. Plasmids were released from the complexes by dextran sulphate (0.1 mg/μg DNA) and their integrity characterized by gel electrophoresis. As control nontreated DNA and non-complexed DNA treated with nuclease were used.

polymer (Fig. 4). The 5 kDa pDADMAC as well as the 24 mol% copolymer were insufficient in protecting DNA even in complexes with charge ratio 1.85. In contrast, the main portion of the plasmid in complexes with charge ratio 1.85 was preserved against enzymatic degradation using the 244, 135 and the 45 kDa pDADMAC, increasingly with increasing molecular weight (Fig. 4A). The complexes with coDADMACs were found to be more effective in stabilizing the plasmid with increasing charge density (Fig. 4B). Nev-

ertheless, changes in the topology of the supercoiled form of DNA could not be prevented. According observations were made after 15 min and 2 h nuclease incubation (data not shown).

Conclusively, an excess of polycation resulted in decreased mechanical or enzymatic degradation of DNA to low molecular weight products which was more effective with high molecular weight and high charge density of the polycations as well as with increased charge ratios of the complexes. The increased stability against degradative mechanisms was found to be correlated with high compaction of the DNA by the polycations. This may reflect stronger individual intermolecular associations, or could result from effective physical shielding of the DNA by DADMACs with high molecular weights or high charge densities.

### 3.4. In vitro cytotoxicity testing

The in vitro cytotoxicity of the polymers and their complexes was measured as a function of time, polymer concentration and complex composition using LDH- and MTT-assay.

Using the LDH-assay the membrane damaging effects of polymer/DNA complexes on L929 cells at three different charge ratios were quantified after 4 h (Fig. 5). Complexes with charge ratio 1.85 were chosen according to the stability experiments described above and complexes with ratio 3.7 were in range where tight condensation occured. Since for low molecular weight polycations such as polyethylenimine (Fischer et al., 1999) an excess of polycation was found to be necessary for efficient transfection, additionally complexes with charge ratio 11.1 were selected. Under these conditions the three copolymers did not induce significant LDH-release (<10%) with exception of the 75 mol% coDADMAC showing a slightly increased LDH-release at charge ratio 11. For the pDADMACs an increase in membrane lysis was found with higher charge ratios of the complexes presumably due to the excess of polymer acting like free polymer. A correlation between polymer molecular weight and cytotoxicity was found by linear regression of log molecular weight versus percentage of LDH-release which gave a correlation coefficient of 0.99 (Fig. 5, insert).

The investigation of the metabolic damaging effects of the free, noncomplexed polycations by MTT-assay reflects the worst case scenario (Figs. 6 and 7). Results from the L929 fibroblasts showed that cytotoxicity was increased with higher incubation times and polymer concentrations in the range from

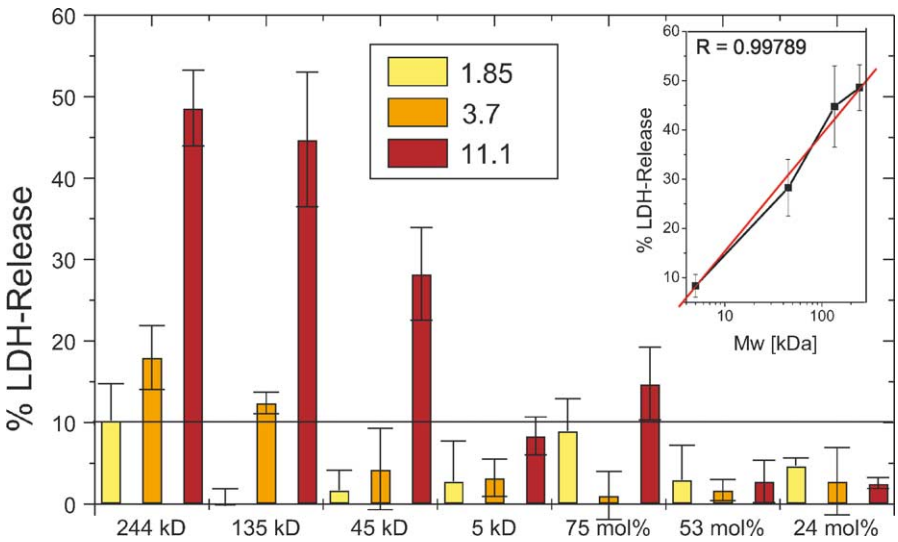


Fig. 5. Membrane damaging effects of herring sperm/polymer DNA complexes. The release of the cytoplasmic lactate dehydrogenase of L929 cells was quantified after 4 h incubation with complexes of three different charge ratios. Results are shown as mean ± S.D. of three experiments. For the pDADMAC homopolymers the log of the molecular weights were correlated with the percentage LDH-release (insert).

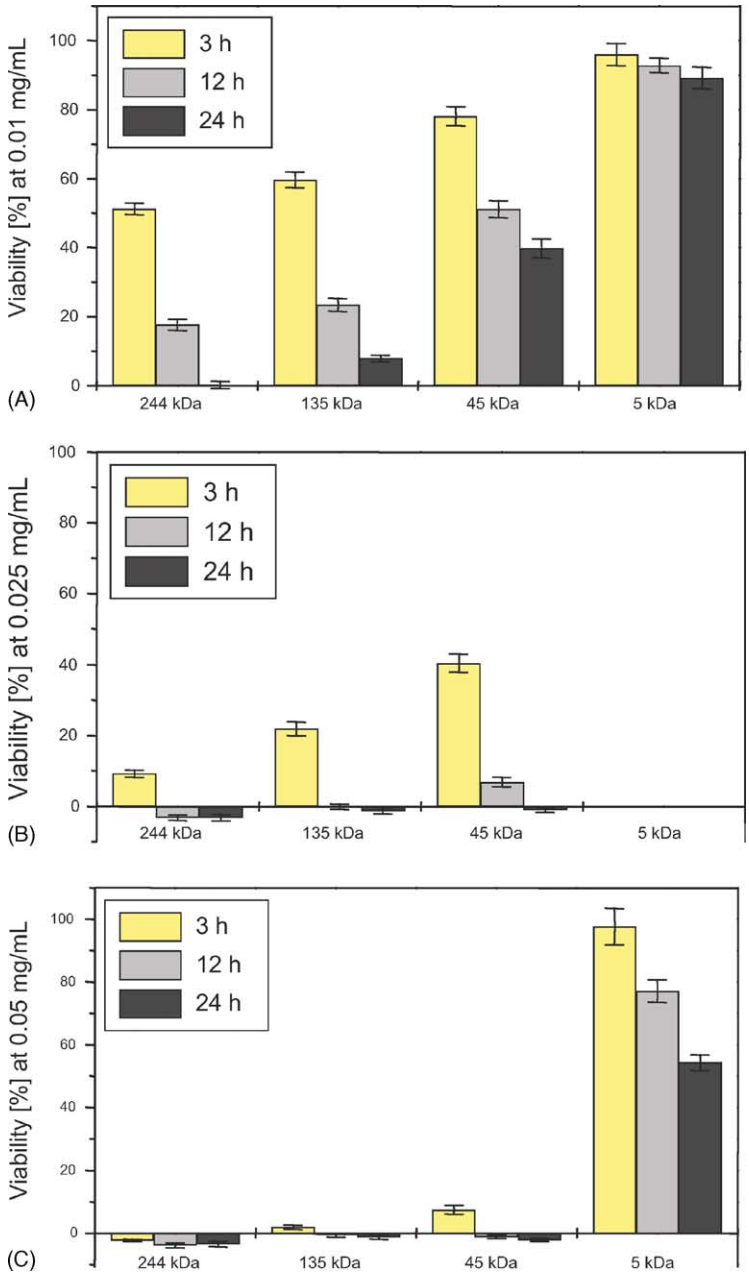


Fig. 6. Dose and time dependent cytotoxic effects of non-complexed DADMAC homo-polymers at 0.01 mg/mL (A), 0.025 mg/mL (B), and 0.05 mg/mL (C). The cell viability was determined by MTT-assay after 3, 12 and 24 h incubation and was shown as mean ± S.D. of seven determinations.

0.001 to 1 mg/mL over 3, 12 and 24 h for all polymers tested. Furthermore, an increase of cytotoxicity as a function of molecular weight as well as charge density could be observed. Significant differences between the four homo-polymers were detectable up to 0.05 mg/mL demonstrating higher cell damaging effects with increasing molecular mass of the polymer (Fig. 6). Higher concentrations of all homo-polymers

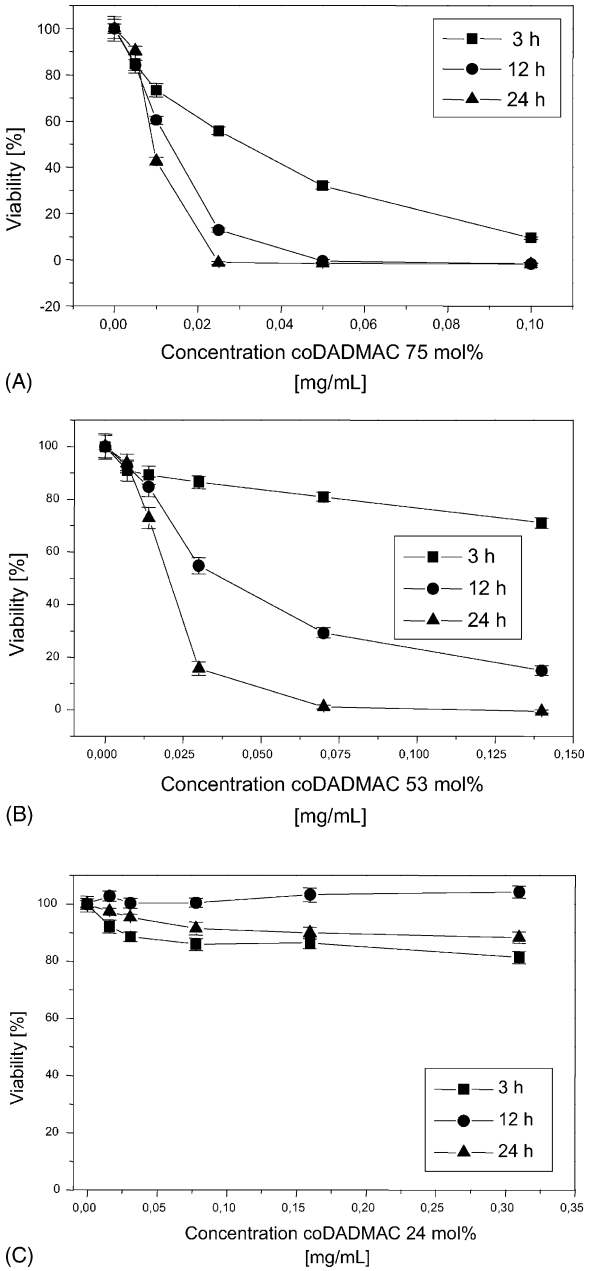


Fig. 7. Evaluation of the time and concentration dependent in vitro cytotoxicity of the non-complexed copolymers with 75 (A), 53 (B), and 24 (C) mol% DADMAC. The influence of charge density of the coDADMACs on in vitro cytotoxic effects on L929 fibroblasts was measured after 3, 12 and 24 h incubation time using equivalent numbers of charges. The cell viability was quantified by MTT-assay. Each point represents the mean ± S.D. of seven determinations.

reduced the cell viability nearly to zero with exception of the 5 kDa pDADMAC. The copolymers were characterized by an increase of the in vitro cytotoxicity with growing charge densities (Fig. 7). Since the same number of cationic charges was used for comparison of the three coDADMACs (see: different concentrations on *x*-axes) only the density of charges should be responsible for the differences in toxicity. Changes in cell morphology observed by phase contrast microscopy were consistent with the results of the MTT-assay (data not shown).

Polycations can induce cytotoxic effects due to electrostatic interactions with negatively charged cell membranes depending on the number, density and arrangement of cationic charges in the three-dimensional structure of the polymer (Fischer et al., 2003). The more charges due to high molecular weights and the higher the density of charges, the higher were the metabolic and membrane damaging effects and subsequent cell death. Our observations were confirmed by the results reported for polyethylenimines (Bieber and Elsaesser, 2001), and poly(methacrylates) (Wolfert et al., 1999; van de Wettering et al., 2000). Compared to other polycations which were successfully used for gene transfer, the 244, 135 and 45 kDa pDADMACs were comparable to polymers like high molecular weight polyethylenimine and poly-L-lysine with regard to in vitro cytotoxicity.

### 3.5. Transfection efficiency

The potential of two pDADMACs (45 and 244 kDa) for introducing DNA into eukaryotic cells was examined. L929 fibroblasts were transfected with pGL3/pDADMAC complexes of different charge ratios (3.75, 11.25 and 22.5). The ratios 3.75 and 11.25 were chosen according to the in vitro cytotoxicity assay. Since for some polymers a high excess of polycation in the complexes were found to be necessary for efficient transfection (Fischer et al., 1999), we tested additionally the higher ratio 22.5. Under the chosen conditions no increased levels of luciferase expression compared to control cells (DNA without polymer) could be observed (Fig. 8). Furthermore, with exception of the complexes with charge ratio 3.75, the vector systems were found to be highly cytotoxic for the cells as observed by microscopic observation. Corresponding to the results of the in

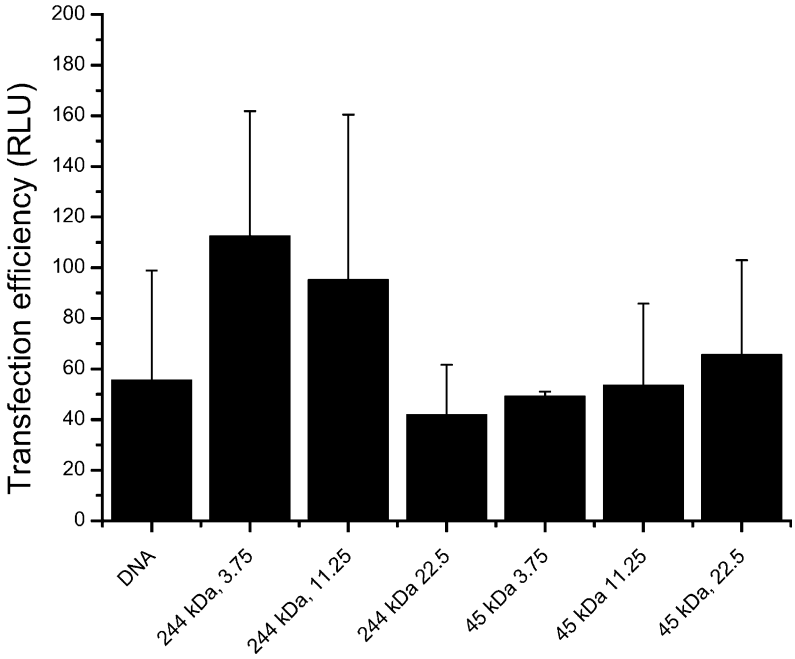


Fig. 8. Transfection efficiency of 244 kDa and 45 kDa pDADMAC on L929 fibroblasts at different complex charge ratios. Values are shown as means ± S.D. of three experiments.

vitro cytotoxicity assay, the 45 kDa induced less cell damage compared to the 244 kDa pDADMAC. The presence or absence of serum or chloroquine did not change the results significantly (data not shown).

The transfection efficiency of all DADMACs was low compared to other gene delivery systems. Low transfer rates were also reported for other polymers with quaternized amines. Similar to DADMACs quaternized poly-(methacrylate)-based polymers showed efficient complex formation but poor transfection (Wolfert et al., 1999; Arigita et al., 1999). The absence of pH responsiveness promoting the endosomal/lysosomal release of the plasmids were hold responsible for this effect. However, since also the addition of the lysosomotropic agent chloroquine did not significantly increase the expression of luciferase by DADMAC polymers, in our experiments the reason must be somewhat different. Influence of serum can also be excluded since transfection results were comparable in the absence and presence of serum. Complex size as key factor for uptake and cytoplasmatic transport of complexes can be neglected since all polymers formed complexes of comparable size. However, for the formation of stable particles a high excess of polycation was found to be necessary which may compromise and limit protein expression due to in vitro cytotoxicity as demonstrated by MTT- and LDH-assay. The issue of cell uptake and intracellular processing requires further studies. Lower tolerance of DNA/pDADMAC as well as DNA/methacrylate derivatives to destruction by added salt compared to polycations with primary amines presumably due to the bulky form of the quaternized groups weaking electrostatic interactions with DNA as well as transfection efficiency was reported (Izumrudov et al., 1999). A critical step might be the capability of dissociation of the DNA from the complexes as also observed by others (Arigita et al., 1999). For quarternized methacrylate derivatives higher DNA-binding affinities and subsequent lower release of DNA under physiological conditions compared to polycations with primary and secondary amines have been described (Wolfert et al., 1999).

### 3.6. Conclusion

In conclusion, the DADMAC polymers are an interesting tool to study structure-activity-relationship with regard to molecular weight and charge density since both structural factors can be systematically modified. With increasing molecular weights and charge densities of the polymers the complexation of DNA, the stability of the complexes and the protection of DNA against enzymatic and mechanical damage were promoted. However, simultaneously with increased electrostatic interactions with DNA also higher charge dependent interactions with cell membranes were observed resulting in higher in vitro cytotoxicity of the polycations and their complexes. Due to the toxicity profiles and the low release of DNA from the complexes, the transfection efficiency was found to be low compared to other polycationic vehicles.

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## References

Arigita, C., Zuidam, N.J., Crommelin, D.J.A., Henninck, W.E., 1999. Association and dissociation characteristics of polymer/DNA complexes used for gene delivery. Pharm. Res. 16, 1534–1541.

Bieber, T., Elsaesser, H.P., 2001. Preparation of a low molecular weight polyethylenimine for efficient cell transfection. Biotechniques 30, 74–81.

Brand, F., Dautzenberg, H., 1997. Structural analysis in interpolyelectrolyte complex formation of sodium polystyrenesulfonate and diallyldimethylammoniumchloride-acrylamide-copolymers by viscosimetry. Langmuir 13, 2905–2910.

Chu, B., 1991. Laser Light Scattering, 2nd ed. Academic Press, Boston.

Dautzenberg, H., Goernitz, E., Jaeger, W., 1998. Synthesis and characterization of poly(diallyldimethylammonium chloride) in a broad range of molecular weight. Macromol. Chem. Phys. 199, 1561–1571.

Dautzenberg, H., Jaeger, W., 2002. Effect of charge density on the formation and salt stability of polyelectrolyte complexes. Macromol. Chem. Phys. 203, 2095–2102.

De Smedt, S.C., Demeester, J., Hennink, W.E., 2000. Cationic polymer based gene delivery systems. Pharm. Res. 17, 113–126.

Felgner, P.L., Barenholz, Y., Behr, J.P., Cheng, S.H., Cullis, P., Huang, L., Jessee, J.A., Seymour, L., Szoka, F., Thierry, A.R., Wagner, E., Wu, G., 1997. Nomenclature for synthetic gene delivery systems. Hum. Gene Ther. 8, 511–512.

Fischer, D., Bieber, T., Li, Y.X., Elsässer, H.P., Kissel, T., 1999. A novel nonviral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm. Res. 16, 1273–1279.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Gebhart, C.L., Sriadibhatla, S., Vinogradov, S., Lemieux, P., Alakhov, V., Kabanov, A.V., 2002. Design and formulation of polyplexes based on pluronic-polyethyleneimine conjugates for gene transfer. Bioconjug. Chem. 13, 937–944.

Godbey, W.T., Barry, M.A., Saggau, P., Wu, K.K., Mikos, A.G., 2000. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J. Biomed. Mater. Res. 51, 321–328.

Howard, A.H., Dash, P.R., Read, M.L., Ward, K., Tomkins, L.M., Nazarova, O., Ulbrich, K., Seymour, L.W., 2000. Influence of hydrophilicity of cationic polymers on the biophysical properties of polyelectrolyte complexes formed by self-assembly with DNA. Biochim. Biophys. Acta 1475, 245–255.

Hubbe M., Mini-encyclopedia of papermaking wet-end chemistry: polydiallyldimethyl-ammoniumchloride (DADMAC), http://www4.ncsu.edu/∼hubbe/DADM.htm, 2003.

Izumrudov, V.A., Zhiryakova, M.V., Kudaibergenov, S.E., 1999. Controllable stability of DNA-containing polyelectrolyte complexes in water-salt solutions. Biopolymers 52, 94–108.

Jaeger, W., Gohlke, U., Hahn, M., Wandrey, C., Dietrich, K., 1989. Synthesis and application of flocculating agents. Acta Polym. 40, 161–170.

Jones, N.A., Hill, I.R., Stolnik, S., Bignotti, F., Davis, S.S., Garnett, M.C., 2000. Polymer chemical factor is a key determinant of physicochemical and colloidal properties of polymer-DNA complexes for gene delivery. Biochim. Biophys. Acta 1517, 1–18.

Kunath, K., von Harpe, A., Fischer, D., Petersen, H., Bickel, U., Voigt, K., Kissel, T., 2003. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. J. Control. Rel. 89, 113–125.

Mahato, R.I., Furgeson, D.Y., Maheshwari, A., Han, S., Kim, S. W., 2000. In: Park, K.D., Kwon, I.C., Yui, N., Jeong, S.Y., Park, K. (Eds.), Polymeric Delivery for Cancer Treatment. Biomaterials and Drug Delivery Toward New Millenium. Han Rim Wan Publishing Co., Seoul, Korea.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., Wagner, E., 1998. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther. 5, 1425–1433.

Plank, C., Tang, M.X., Wolfe, A.R., Szoka, F.C., 1999. Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes. Hum. Gene Ther. 10, 319–332.

Ruppelt, D., Kötz, J., Jaeger, W., Friberg, S.E., Mackay, R.A., 1997. Influence of cationic polyelectrolytes on structure formation in lamellar liquid crystalline systems. Langmuir 13, 3316–3319.

Tang, M.X., Szoka, F.C., 1997. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther. 4, 823–832.

van de Wettering, P., Schuurmans-Nieuwenbroek, N.M.E., van Steenbergen, M.J., Crommelin, D.J.A., Henninck, W.E., 2000. Copolymers of 2-(dimethylamino)ethyl methacrylate with ethoxytriethylene glycol methacrylate or *N*-vinyl-pyrrolidone as gene transfer agents. J. Control. Rel. 64, 193–203.

Wolfert, M.A., Dash, P.R., Nazarova, O., Oupicky, D., Seymour, L.W., Smart, S., Strohalm, J., Ulbrich, K., 1999. Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. Bioconjugate Chem. 10, 993–1004.

Zelikin, A.N., Putnam, D., Shastri, P., Langer, R., Izumrudov, V.A., 2002. Aliphatic ionenes as gene delivery agents: elucidation of structure-function relationship through modification of charge density and polymer length. Bioconjugate Chem. 13, 548–553.

Zimm, B.H., 1948. The scattering of light and the radial distribution function of high-polymer solutions. J. Chem. Phys. 16 (12), 1093–1099.