Performance of three PDMAEMA-based polycation architectures as gene delivery agents in comparison to linear and branched PEI

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1. Introduction

In order to genetically modify (‘transfect’) a eukaryotic cell, the recombinant DNA has to enter the cell, i.e. pass the cellular membrane, and then reach and finally enter the nucleus, where transcription takes place [1]. Viruses achieve this very well and therefore are popular transfection agents in medicine. In biotechnology, however, non-viral transfection agents are preferred, inter alia for reasons of safety. The first step in non-viral transfection is the compaction of the negatively charged DNA with a cationic agent, such as a lipid or polymer. In case of the use of cationic polymers (‘polyfection’), the ensuing complex (‘polyplex’) should bear a net positive charge in order to be attracted to the negatively charged cell surface. Independent of the agent used, the success rate (‘transfection efficiency’) of the non-viral protocols is at present orders of magnitude lower than that of the viral ones, presumably due to bottlenecks encountered by the DNA on its way to the nucleus.

Our understanding of DNA compaction in polyelectrolyte complexes is well developed and effects such as size and charge density of the polycation, e.g. on complex stability and size, can be explained by our current understanding of physical chemistry of polyelectrolytes. When the biological effect, i.e. the transfection capability, of such complexes is compared, however, one polycation, namely polyethylenimine (PEI) and some copolymers thereof, shows vastly superior performance, although still much less than any viral agent. The molecular basis for this difference to other polycations has been speculated upon (see below), but is still far from clear, in particular in regard to the intracellular events. However, aside some isolated exceptions (e.g. Refs. [2,3]), most of the hitherto investigated polycations such as, e.g., PDADMAC or PDMAEMA show transfection efficiencies in the single digit percent range [4–6]. Some authors report a rough correlation between cytotoxicity of a transfection agent and its transfection capability (e.g. Refs. [7,8]), others have correlated transfection efficiencies of synthetic polycations with their size [4,9], or the stability of the polyplex in solution [10]. The structure of the polymer, e.g. linear, branched, or star-shaped, also seems to make a difference in transfection efficiency (e.g. Ref. [3]).

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**Abstract**

Polycations such as PEI (poly(ethylenimine)) and PDMAEMA (poly(2-dimethylamino) ethyl methacrylate) are commonly used as non-viral gene delivery vehicles. Differences in efficiency exist, but comparative experiments on DNA compaction, cellular uptake, cellular trafficking and finally nuclear entry are largely lacking. In this study, ATRP (atom transfer radical polymerization) was used to synthesize three highly defined structures of PDMAEMA (linear, highly-branched, and star-shaped), which were systematically compared to linear and branched PEI. For the investigation of intracellular trafficking, polymers were fluorescently labelled. The ability to compact DNA and cellular uptake efficacy were similar for all investigated polycations. Transfection efficiencies at optimized N/P ratio showed similar trends as cytotoxicities of the free polycations, with the star-shaped PDMAEMA reaching almost similar orders of transfection efficiencies as branched PEI. Twenty-four hours post-transfection, polymer fluorescence was almost exclusively found in close proximity (but never inside) the nucleus. In case of the ‘good’ transfectants (PEI, star-shaped PDMAEMA), the fluorescence concentrated in ‘spots’ of high intensity, arguing for localization in specific sub-cellular structures.

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Not surprisingly, PEI, as the most powerful non-viral transfection agent, has been subject of a much greater number of studies than other polycations, in particular in regard to the intracellular trafficking. According to these studies, entry of the PEI-based polyplexes into the cell seems to occur by unspecified as well as clathrin- and caveolae-dependent endocytosis [11]. Such an uptake mechanism means that in order to reach the nucleus, the DNA has to escape from the endosome [12]. Several authors have thus linked the superior performance of PEI to the ‘proton sponge’ effect, i.e. the ability of PEI to buffer the acidic environment found in endo- and lysosomes, thereby increasing the water influx into the vesicle and finally damaging the vesicle membrane to allow DNA escape, see e.g. Akinc et al. [13] and Bieber et al. [14] for a rigorous investigation of the involved mechanisms.

Once escaped from the endosome, the DNA has to travel through the cytosol to the nucleus. Even in the case of PEI little is known about the mechanism of this process. However, several authors describe that polyplexed PEI, but also the naked polycation, shows nuclear localization within a few hours post-transfection (e.g. Refs. [14,15]). According to these experiments, only a small fraction of the originally entering DNA reaches the nucleus. Finally, in order to actually enter the nucleus, the DNA has to overcome the nuclear membrane, respectively, await cell division, a moment during which the nuclear membrane disintegrates.

Compared to PEI, the performance of most alternative polyacations is less well documented on a molecular/mechanistic level. Van der Aa et al. [11] were able to show that the uptake routes for PEI and PDMAEMA may be different, while the better transfection efficiency of PEI might simply be related to the larger size of the PEI-DNA-polyplexes. Most of the time, however, differences in transfection efficiency are discussed within the hermeneutics of the above-mentioned proton sponge hypothesis. It has been shown that the addition of endosome disrupting agents is improving transfection outcomes for PDMAEMA and other polycationic methacrylates, however, no rigid proof for the modus operandi was presented [16]. Jones et al. [17] have recently shown that PDMAEMA, contrarily to earlier reports, is not able to physically disrupt the late phase endosome, although changes in the morphology of the organelle occur.

The lack of studies comparing directly the intracellular demeanour of PEI and other polycations renders the interpretation of the performance, but also its improvement difficult. Here we present a systematic investigation comparing PEI- and PDMAEMA-based transfection involving several polymer structures (linear, branched, and star-shaped), which should be conductive to a re-evaluation of the potential bottlenecks to DNA delivery using polycations. By necessity our experiments are thus restricted to conditions, reagents, and protocols suitable for transfection.

2. Experimental

2.1. Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33258, and branched polyethylenimine (25 kDa) were from Sigma Aldrich (Taufkirchen, Germany). Linear PEI (25 kDa) was from Polysciences, Inc. (Warrington, Pennsylvania, USA), Rhodamine B ethylenediamine, and Rhodamine red X succinimidyl ester were from Invitrogen GmbH (Hamburg, Germany). PEls were prepared as 10 μM aqueous stock solutions, all other polyners as 500 μM stock solution. Cell culture materials were from Greiner Bioscience (Frickenhausen, Germany). Cell culture media and solutions were from PAA (Colbe, Germany). Serum reduced medium OptiMEM was from Invitrogen. Plasmid DNA was prepared by using the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). Milli-Q water was used for the preparation of all aqueous solutions.

2.2. Plasmid DNA

Plasmid pH2B-eGFP (5.1 kb) [18], encoding for the nuclei localized GFP (green fluorescent protein), was used in all transfection experiments. The plasmid was amplified in Escherichia coli DH5 alpha strain in LB medium to sufficient quantities by using standard molecular biology techniques, including harvesting and purification via Qiagen’s Maxi- or Giga-Prep kits. Plasmid DNA (pDNA) concentration and quality were determined by A260/280 ratio and by agarose gel electrophoresis.

2.3. Polycation synthesis and labelling

The linear (17 kDa), star-shaped (59 kDa) and highly-branched PDMAEMA (11.6 kDa) polymers were synthesized according to previously reported procedures [19,20]. Linear PEI was labelled with Rhodamin B by incubating 8 μmol of polymer with 16 μmol of Rhodamin B 17.6 μmol N,N-dicyclohexylcarbodiimide (DCC) and 0.5-1.0 mg of 4-(dimethylamino)-pyridine (DMAP) for 24 h at room temperature in 20 mL N,N-dimethylacetamide (DMAC). Unreacted rhodamine was removed by exhaustive dialysis against pure Milli-Q water. Fluorescent labelling of the branched PEI with Rhodamine red X was performed as described previously [21]. In case of PDMAEMA, dye labelling with Rhodamine B was achieved by reacting a diethyleneamine-functionalized Rhodamine B derivative with the terminal bromine functions of the PDMAEMA polymers. The extent of labelling was semi-quantified by optical determination of the fluorescence intensity provided by a standardized amount of polymer (reader: Genios Pro, Tecan GmbH, Crailsheim, Germany). In a typical reaction 2.29 × 10⁻⁶ mol of PDMAEMA and 2.38 × 10⁻⁶ mol of rhodamine were dissolved in 5 mL of DMF and placed in a 25-mL-flask equipped with a magnetic stir bar. The flask was then filled with a rubber septum and the content degassed by bubbling nitrogen for 15 min. The reaction mixture was stirred at 50 °C for 48 h. Unreacted rhodamine was removed by exhaustive dialysis against Milli-Q water. After freeze-drying, the purified product was obtained as a pink powder.

2.4. Mammalian cell line and culture conditions

The Chinese Hamster Ovary CHO-K1 (CCL-61, ATCC) cell line used in the transfection experiments was maintained in RPMI 1640 cell culture medium supplemented with 10% FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2 mM l-glutamine. Cells were cultivated at 37 °C in a humidified 5% CO₂ atmosphere.

2.5. Interpolyelectrolyte complex-based transfection of mammalian cells

For transfection cells were seeded at a density of 2 × 10⁵ cells/well in 6-well plates 20 h prior transfection. One hour prior transfection, cells were rinsed with PBS and supplemented with 2 mL OptiMEM. pDNA/polymer polyplexes were prepared at room temperature using 3 μg pDNA together with varied amounts of the respective polycation stock solution to achieve the indicated nitrogen/phosphate (N/P) ratios. For this purpose, the pDNA solution and the mixture was immediately vortexed for 10 s at full speed, followed by incubation at ambient temperature 20 min (formation of the IPECs). The polyplex suspension (200 μL) was added to the cells and the plates were centrifuged for 5 min at 200 g and placed for 4 h in the incubator. Afterwards, the medium was re-
moved by aspiration, 2 mL of fresh growth medium were added, and the cells were further incubated for 20 h. Cells were harvested by trypsinization and resuspended in PBS. Dead cells were identified via counterstaining with propidium iodide. The relative expression of eGFP fluorescence of 1 × 10^6 cells was quantified via flow cytometry using a Cytomics FC 500 (Beckman Coulter, Krefeld, Germany).

2.6. Zeta-potential measurement

For zeta-potential measurements, polyplexes were prepared in 1 mL of a 10 mM aqueous NaCl-solution containing a total of 15 μg pDNA, following otherwise the ‘transfection protocol’ indicated above. Zeta-potential measurements were performed in triplicate using the standard capillary electrophoresis cell of the Zetasizer Nano ZS (Malvern Instruments, Ltd., UK) at room temperature.

2.7. Dynamic light scattering

Polyplex sizes were measured by dynamic light scattering (DLS) using an instrument equipped with a compact goniometer (ALV DLS/SLS-SP 5022F), an ALV 5000/E cross-correlator and a He–Ne laser (λ₀ = 632.8 nm, 20 mW). One milligram pDNA in 1 mL of aqueous 150 mM NaCl-solution was mixed with various amounts of polymer stock solution (bolus addition) to reach the indicated N/P ratios, following by incubation for 20 min at room temperature. Only star-shaped and linear PDMAEMA as well as branched PEI were included in the measurements as the other two polymers precipitated at the concentration required for DLS. Free plasmid and free polymers were measured under similar conditions, at 1 mg/mL and 20 mg/mL, respectively. The particle diameters were quantified via counterstaining with propidium iodide. The relative fluorescence from polyplexes located in the perinuclear and the intranuclear areas.

2.8. MTT assay

The cytotoxicity of the cationic polymers was evaluated in 96-well microtitre plates by the MTT assay [22]. For this, CHO-K1 cells were seeded at a density of 1 × 10^4 cells/well 20 h prior to the experiment. Then, they were incubated with the complexes or the free polymers for 4 and 20 h (after medium change from serum-free to serum-containing medium, see transfection protocol above). After incubation, cells were rinsed with PBS and further incubated in 200 μL MTT solution (0.5 mg/mL in PBS) for 2 h. The solution was aspirated, replaced with 200 μL DMSO and mixed at 150 rpm for 5 min to dissolve the formazan crystals produced in the reaction. Absorbance was then measured at 580 nm in a microplate reader (Genios Pro, Tecan GmbH, Crailsheim, Germany) with untreated cells serving as controls.

2.9. Microscopy

Epifluorescence and confocal laser scanning fluorescent microscopy were performed to visualize the intracellular localization of the polyplexes. CHO-K1 cells were inoculated at a density of 2 × 10^5 cells/well in 6-well plates, 20 h prior transfection. Rhodamine labelled polymer solutions (PEIs: 10 μM; PDMAEMAs: 500 μM) were mixed to 3 μg of plasmid DNA in aqueous 150 mM NaCl-solution (final volume 200 μL) at a N/P ratio of 5 or 10. After 20 min incubation at room temperature, the polyplexes were added to the cells. After 24 h of incubation, the cells were washed twice with PBS, fixed with 4% p-formaldehyde solution for 20 min and washed again twice with PBS. Then, the fixed cells were counter-stained for 15 min using 1 μg/mL nuclear dye Hoechst 33258 in PBS. Cells were washed twice with PBS and once with Milli-Q water. The cover slips were mounted in Prolong Gold mounting medium (Invitrogen) according to the manufacturer instructions. Cells were observed under an epifluorescence microscope (Olympus BX51TF, Hamburg, Germany). In addition, images were obtained using a laser scanning confocal microscope (Leica TCS-SP, Leica, Wetzlar, Germany) equipped with argon and neon lasers. Fluorescence images were captured and processed with a digital imaging processing system (Leica TCS software, Leica, Wetzlar, Germany). The cells were scanned in three dimensions as a z-stack of two-dimensional images (1024 × 1024 pixels). An image cutting horizontally through the cell at approximately mid-height was selected out of a z-stack of images to distinguish the fluorescence from polyplexes located in the perinuclear and the intranuclear areas.

2.10. Statistical analysis

Group data are reported as mean ± SD. For transfection results, the Student’s t-test was used to determine whether data groups differed significantly from each other. Statistical significance was defined as having P < 0.05.

3. Results and discussion

3.1. Polymer synthesis and dye labelling

Linear, star-shaped and highly-branched PDMAEMA were synthesized utilizing ATRP (atom transfer radical polymerization) according to procedures described previously by some of our group [19,20]. The star-shaped polymers are based on a glucose core as initiator, giving rise to a potential maximum of five arms per star. However, due to non-quantitative initiation, this synthesis yields an average amount of 3.1 arms per molecule [20]. The highly-branched PDMAEMA was synthesized by copolymerizing an inimer (initiator + monomer) in a ratio of monomer to inimer of 10. This leads to an average of one branching point for every eleventh monomer incorporated within the polymer chain. The dye labelling of these polymers was accomplished in an extremely facile fashion by substituting the terminal bromine groups with a commercially available amine functionalized Rhodamine B derivative. The excess of unreacted dye could easily be removed by exhaustive dialysis, yielding highly fluorescing polymers. Average molecular weights (Mₙ) and polydispersities of all polymers used in this investigation are compiled in Table 1.
3.2. Complexation of DNA by the various polycations

The goal of our research was to better understand the performance of polycations as transfection agents, i.e. in the context of the genetic modification of mammalian cells using polycation-DNA-interpolyelectrolyte complexes (IPECs). In consequence, our experiments focused on conditions, reagents, and protocols proven suitable for transfection purposes. For instance, IPEC-formation in all experiments took place under conditions mirroring those of the original PEI-based transfection protocol, albeit at varied N/P ratios.

The polymer-DNA IPECs (polyplexes) were first characterized by dynamic light scattering (DLS) and cryogenic transmission electron microscopy (cryo-TEM). In addition, the fact that all polyplexes bore a positive net charge (necessary for interaction with the negatively charged cell membrane und cellular uptake) was verified by zeta-potential measurements. The DLS intensity-weighted distribution functions of the hydrodynamic radii for the complexes of plasmid DNA with branched PEI, star-shaped PDMAEMA and linear PDMAEMA are displayed in Fig. 1. The data were evaluated with the CONTIN algorithm, which allows the simultaneous detection of various size species in the highest resolution possible. Note that a cumulant analysis, i.e. a one particle fit, is inappropriate to characterize such systems.

All samples show a distribution characterized by the presence of three peaks at different hydrodynamic radii. The peak corresponding to the smallest sizes, just below 10 nm, originates from individual and non-complexed polymer molecules; note that free diffusing DNA molecules in solution would have a \( R_h \) of 50–56 nm. The second (central) peak in the distribution curves corresponds to the majority of the IPECs. The average values for this peak \( \langle R_h \rangle = 45–65 \text{ nm} \) are in the same order as those obtained for freely diffusing DNA in solution \( \langle R_h \rangle = 50–56 \text{ nm} \), we can thus deduce that typically a single DNA molecule is complexed by the polymers. The third peak in the distribution curve corresponds to fairly large structures with radii in the range of 1 \( \mu \text{m} \) and is caused by a very minor, yet always detectable fraction of larger polyplexes. The actual weight and number fraction of these complexes is negligibly small, the comparatively large area of the peak is due to the fact that the signal intensity scales with \( R_h^6 \).

In this context, it should be acknowledged that DLS measurements can be problematic in case of particles above 1 \( \mu \text{m} \) (sedimentation) or below 10 nm (accuracy). However, we refrained from removing such species from our IPEC-preparation, as we wanted to investigate the typical transfection cocktail supplied to the cells. IPECs are dynamic structures, removing either the larger aggregates or the free polycations may conceivably change the shape and composition of the IPECs. Moreover, extent to which such difficulties manifest themselves most likely is minor in our case. Sedimentation is a function of the density gradient between the particles and the surrounding media, i.e. most likely negligible in the case of highly swollen polyelectrolyte complexes considered here. Additionally, the IPECs are charged, which also counteracts sedimentation. Concerning the free polycations in the preparation, our group has previously published repeatedly on the use of light scattering for the characterization of similar star-shaped polyelectrolytes, nanoparticles, and other tiny structures and shown that

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<td>Molecular weight ( M_n ) and polydispersity index (PDI) of the polymers used in this investigation.</td>
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<tr>
<td>( M_n ) (g/mol)</td>
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<tr>
<td>Linear PDMAEMA</td>
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<td>Star-shaped PDMAEMA</td>
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<td>Linear PEI</td>
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<td>Branched PEI</td>
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* No data available.
** According to the manufacturer.
the instrument in question is able to resolve such structures with sufficient precision.

A closer look at the evolution of the distribution curves as a function of the N/P ratio shows that the fraction of free polymer increases steadily with rising N/P ratio. However, we can observe a difference in the development depending on the type and architecture of the polymer. Whereas branched PEI and the linear PDMAEMA show significant fractions of free polymer only for the highest N/P ratios, the star-shaped PDMAEMA polymer already exhibits considerable fractions of free polymer for rather low N/P ratios. From this it can be deduced that a fairly low amount of star-shaped PDMAEMA is necessary to efficiently complex a given amount of DNA. In addition, branched PEI shows mainly large aggregates for an N/P ratio of 1, as previously reported, e.g., by Wen et al. [23] and the addition of more polymer is necessary to convert the initially formed large complexes, comprising several DNA molecules, into smaller structures.

The average hydrodynamic radii of the complexes show little dependency on the N/P ratio, with average values over all N/P ratios of 60, 53.5, and 50 nm for branched PEI, star-shaped and linear PDMAEMA, respectively. The radii for branched PEI-based complexes are thus slightly larger than for the PDMAEMA based ones. The values scatter in a region of 10% and the only the star-shaped PDMAEMA shows a minor decrease for the polyplex size at larger N/P ratios. The consistency of the complex size for different N/P ratios is an important finding, as previous studies have sometimes suggested a significant decrease of the average hydrodynamic radius with rising polymer content [3]. However, many of the prior experiments were based on zeta-potential measurement devices with no or only poor ability to resolve distribution functions. Herein, we conducted the solution analysis with dynamic light scattering and a high resolution CONTIN fit, which can give far more detailed information. For instance, looking at Fig. 1B, the increasing contribution of the free star polymer to the scattering behaviour would have artificially lowered the average value, if the detailed distribution analysis had not been done.

To gain further insights into the aggregate structures, we performed cryo-TEM measurements of complexes based on branched PEI and star-shaped PDMAEMA, Fig. 2. The N/P ratios of the samples (5 and 10) were chosen based on amounts known to be required for sufficient complexation and/or efficient transfection into CHO cells. The complexes formed by the star-shaped PDMAEMA (Fig. 2A and B) showed a strong tendency to adsorb onto the slightly negatively charged lacey grid. Multiple complexes were often formed as can be seen in both Fig. 2A and B. Moreover, these aggregates appear darker and denser than the aggregates formed by branched PEI (Fig. 2C and D). In addition, complexes based on branched PEI showed no tendency to adsorb to the grid. Aggregate sizes in the cryo-TEM appear somewhat larger than the corresponding apparent hydrodynamic radii determined by DLS. This can be caused by the confinement of the aggregates in the thin film of vitrified water, but also from the fact that aggregate shape is not well approximated by the hard sphere model assumed in the DLS analysis. The looser appearance of the branched PEI-DNA complexes can also be seen in the DLS analysis, which showed larger hydrodynamic radii for branched PEI-DNA complexes than for the PDMAEMA-DNA ones.

3.3. Cytotoxicity studies

Toxicity is a major issue with many non-viral vectors and a rough correlation between toxicity and transfection efficiency has been described in the past (e.g. Ref. [1]). Here, MTT assays were performed to evaluate the metabolic activity of CHO-K1 cells exposed to the PDMAEMA and PEI polymers for 4 h in OptiMEM medium and for 20 h in serum-containing medium to mimic transfection conditions. For data normalization, control cells were treated analogously but without the addition of the polymers. The data are compiled in Fig. 3. Some of the viabilities shown in Fig. 3 surpass the value of 100%. This is due to the experimental set up, in particular to the choice of the controls. Cell growth in 96-well plates is known to be highly depending on the position.
In order to be consistent we always cultivated the control cells at the same positions in the plate and normalized our results to the viabilities found there. This leads to values above 100% for the relative cell viability in some of the wells.

As shown in Fig. 3A, after 24 h incubation, all polycations were characterized by a concentration-dependant cytotoxicity. The star-shaped PDMAEMA, branched PEI, and linear PEI exhibited the highest toxicities with IC50 values of 6–8 µM, whereas the linear and highly-branched PDMAEMA showed a 4-fold weaker toxicity with an IC50 value of 30 µM (Fig. 3A). As previously described [7], the cytotoxicity of the polycations correlates with the molecular weight (see also Table 1), with smaller molecules causing lower cytotoxicities.

Additionally, the non-lethal N/P ratio of the various polyplex types for the chosen cell line was determined prior to the actual transfection experiments. In essence, the experiments were performed as the cytotoxicity studies described above, except that instead of incubating the cells with polymer solutions, polyplexes were used. Using untreated cells as control, the CHO-K1 cells were exposed to N/P ratios between 1 and 240. The DNA concentrations in these experiments were always 15 µg/mL and the polymer concentrations were adjusted according to the indicated N/P ratio.

After 4 h of incubation, the observed effect on the mitochondrial activity showed the same trends as for the corresponding free polymers, Fig. 3B. However, whereas the toxic effect exerted by the branched PEI-based polyplexes increased above N/P ratio of 10, the toxic effect of the polyplexes based on PDMAEMA or linear PEI manifested itself only above N/P ratios of 12 and 20. Among the PDMAEMA-based structures, polyplexes based on linear PDMAEMA displayed the highest toxicity, while the star-shaped PDMAEMA displayed the lowest.

Comparing our results to previously published data, the linear and highly-branched PDMAEMA used in this study are less toxic than the polymers investigated in previous studies, although similar trends in cytotoxicity as a function of structure are seen [6,7,16,25]. Fischer et al. [7] found that besides the molar mass, the charge density, i.e. a key parameter for the interaction with biological membranes, is most decisive for toxicity. Cell death seems to be by necrosis in most cases [17]. Most recently Xu et al. [3] also showed that in the case of PDMAEMA a star-shaped
architecture can actually increase transfection efficiency, while reducing cytotoxicity compared to linear PDMAEMA. However, the effect described by these authors was less pronounced than in our case.

3.4. Transfection efficiency studies

The transfection properties of the PDMAEMA and PEI polymers were evaluated using the H2B–eGFP system, which causes in case of a successful transfection the expression of the green fluorescent protein (GFP) in the nucleus of the cell. This system thus provides a cell-by-cell measure of transfection activity, which can be quantified in a statistical manner by flow cytometry. Using untreated cells as negative control, CHO-K1 cells were transfected with polyplexes of all investigated polycations at N/P ratios between 1 and 12. The average of the transfection efficiencies and the percentage of cell survival were evaluated 24 h after transfection by flow cytometry. Fig. 4. As expected, all polyplexes showed only weak toxicity at the tested N/P ratios (PDMAEMA and linear PEI < 5%; branched PEI < 15%). The number of transfected cells was not significantly different when branched or linear PEI was used as transfection reagent (compare Fig. 4A and B). The transfection efficiencies with the star-shaped PDMAEMA polycation reached an optimum at an N/P ratio of approximately 7, whereas they were comparable to those provided by PEI. PEI transfection efficiencies could be further improved by increasing the N/P ratio. This could correlate to the previously shown DLS data (Fig. 1) according to which a lower amount of star-shaped PDMAEMA is required to efficiently complex plasmid DNA in comparison to branched PEI and linear PDMAEMA. Thus, optimal transfection in the case of PDMAEMA is observed in the non-toxic region, whereas toxic amounts of polyplexes are required for optimal transfection in case of PEI.

PEI is the gold standard among the polymeric gene carriers reported. However, its toxicity remains a concern at least for in vivo application [15]. The star-shaped PDMAEMA might thus offer an alternative to PEI for non-viral gene delivery. It should also be noted that with a standard deviation consistently below 10% the reproducibility of the transfection data is far better for the star-shaped PDMAEMA than for either PEI, where standard deviations ranging between 14% and 20% were found. This may be due to the lower polydispersity of the star-shaped PDMAEMA. In this context, it should be noted that among the PDMAEMA-structures produced for this study the highest polydispersity were determined for the branched PDMAEMA, which may have an influence on the kinetics and mechanistic pathway of the complexation and thus on the final (low) transfection efficiencies of the corresponding polyplexes. Well-defined polymers prepared by ATRP could thus have a beneficial influence for the reproducibility of the process.

In comparison to the star-shaped PDMAEMA, the transfection efficiency of the highly-branched PDMAEMA was 8-fold lower at N/P ratios of 5 and 10. Since these polycations did not exhibit cytotoxicity when complexed with DNA at N/P ratio ≤ 30 (Fig. 3B), we repeated the transfection experiments rising the N/P ratios up to 60. However, this did not improve the transfection efficiency. The mechanistic relationship between cytotoxicity and transfection efficiency is presently unknown, though it also seems to be operative in case of the linear PDMAEMA, where low transfection efficiencies also coincide with low toxicities in the investigated range, i.e. N/P ratios ≤ 12.

3.5. Intracellular trafficking studies

Since the DLS analysis had revealed constancy in IPEC size for all polymers, changes in the transfection efficiencies for various N/P ratios cannot be related directly to differences in the compacted structures. Likewise, the absence of toxicity for linear and highly-branched PDMAEMA does not sufficiently explain the lack of transfection activity at the tested N/P ratios. Therefore, additional experiments focussing on polypep trafficking within the cells were performed to interpret these findings.

For all experiments described below, the fluorescence signal emitted by the labelled polycations was first monitored by epifluorescence microscopy and then by CLSM (confocal laser scanning microscope) to gain more detailed information about the cellular localization of the polycations and thus indirectly of the polyplexes. A preliminary test was performed to exclude that the uptake of the polyplexes was unspecifically driven by the fluorochrome (rhodamine). For this purpose, cells were incubated for 24 h with free rhodamine (final concentration 0.11 μM) and put under the microscope. In all cases, the corresponding red fluorescence was restricted exclusively to the plasma membrane. When the cells were instead incubated with rhodamine-labelled branched PEI (0.7 μM, incubation protocol similar to the transfection protocol), the fluorescence accumulated in vesicle-like structures spread throughout the cytoplasm, which in around thirty percent of the cells showed accumulation close to the nucleus.
Subsequently, we investigated the influence of the fluorescent labelling of the polymers on the transfection efficiency. For this purpose, CHO-K1 cells were transfected in parallel with rhodamine-labelled, Cascade blue-labelled and non-labelled polycations. If anything the transfection efficiency data obtained with rhodamine-labelled polycations were slightly lower than those obtained with non-labelled polymers, indicating that introduction of the hydrophobic dye did not improve DNA uptake. Similar observations have already been reported by others [26]. Cascade blue-labelled PEI showed slightly elevated transfection efficiencies.

In order to get information on the fate of the polycations within the cells, we transfected CHO-K1 cells with linear and star-shaped rhodamine-labelled PDMAEMA (N/P 5) as well as with rhodamine-labelled and Cascade blue-labelled PEI (N/P 10; Cascade blue only branched PEI) and examined the cells 24 h after transfection by epifluorescence microscopy. Transfected cells were recognizable by their green nuclei, caused by the presence of the recombinant, histone-bound eGFP. The examination of the slides at low magnification showed that all cells treated with rhodamine-labelled PEI or PDMAEMA polyplexes displayed red fluorescence of similar intensity, indicating that the efficiency of the uptake of the complexes did not differing significantly between the polymers and therefore most likely was not responsible for the differences in transfection efficiency. Fluorescence was observed to aggregate in ‘spots’, usually found in the vicinity of the nucleus, Fig. 5. The spots in the vicinity of the nucleus showed a distinct tendency to cluster in one or two positions, Fig. 6. Interestingly, spots were maintained even during and after cell division, arguing against polymer entry into the nuclear space at breakdown of the nuclear membrane. This is further supported by the fact that we were not able to see association of polyplexes and the chromosomal DNA in experiments involving tubulin labelling.

In order to exclude that spot formation was simply due to rhodamine unspecifically stained organelle membranes [27], we repeated the experiment with Cascade blue-labelled PEI obtaining essentially the same results. A semi-quantitative analysis of the microscopy pictures for GFP expression, i.e. ‘transfection efficiency’, obtained after transfection with polyplexes based on the different investigated polycations, showed a similar picture to the statistical transfection efficiency data obtained above by FACS, Fig. 4. Thus, cellular uptake and intracellular localization is not necessarily linked to efficient expression of the recombinant gene.

Finally microscopy slides were re-analyzed by CLSM. Since the device did not permit the detection of the Hoechst dye, we used instead the eGFP fluorescence in the transfected cells to localize the nucleus. The confocal microscopy examination of the middle focal plane which traverses both cytoplasm and nucleus of a cell (Fig. 6) revealed that in all cases the polyplexes formed aggregates that were located in the cytoplasm with particular enrichment in the perinuclear region and sometime localization at one pole of the nuclei (Fig. 6B–D). Interestingly, whereas branched PEI, star-shaped PDMAEMA and to a lesser extent linear PEI seem to accumulate in defined sub-cellular structures, the linear PDMAEMA...
led to a diffuse staining in the cytoplasm of eGFP expressing cells. This is demonstrated in Fig. 6D, which shows one of the rare cells that expressed eGFP after transfection with linear PDMAEMA (indicated by the brightly green nucleus).

At this point, we cannot exclude that the aggregation pattern detected for branched PEI, linear PEI and star-shaped PDMAEMA is an artefact of the rhodamine labelling. However, this would not explain the deviating behaviour of the linear PDMAEMA, which carries a similar ratio of fluorescent molecules (as evidenced by optical measurements), nor the similarity of the behaviour of the branched PEI, which carries a much higher label density than the other investigated polycations. Furthermore, the transfection efficiencies with and without labelling are very similar, thus excluding a strong influence of the dye component. The consistent spot-like fluorescent pattern observed for the ‘good transfectors’ instead suggests localization in organelles (i.e., endosomes, lysosomes, calveosomes), which are commonly found in the vicinity of the nucleus [11,13,28], whereas the linear PDMAEMA (and by inference the corresponding polyplexes) either never enters such sub-cellular structures or is more easily released from them. Given the fact that release from the endosomes is usually assumed to present the bottleneck in transfection, the lower transfection efficiency of the linear PDMAEMA remains puzzling.

For the time course analyzed, no labelled polymers could be detected within the nuclei although the pDNA must have entered given the fact that the reporter gene was expressed by these cells. It is possible that the plasmid DNA has been released from the polyplexes prior to transport into the nucleus. Although some degree of pDNA/PEI complex dissociation is likely to occur in the cytoplasm, our inability to detect the polymer within the nucleus could also be related to the detection limit of the fluorescence technique. On the other hand, Godbey and co-workers reported that, a plasmid enters the nucleus mostly as a complex 6 h post-transfection [15]. Bieber and co-workers reported that they observed PEI nuclear localization predominantly during the first 3–6 h of the transfection period and could no longer detect the polymer after 24 h incubation [14]. Thus the time course of the analysis could also play a role in the ability to detect the labelled transfection agent within the nucleus. In order to clarify this, kinetic study of polyplexes uptake and trafficking, comparative investigation in different cell lines and cell fractionation experiments are under way.

4. Conclusion

ATRP polymerization allows the controlled production of polymeric structures. Here it was used to produce three types of PDMAEMA, a linear, a highly-branched and star-shaped one. With these defined molecules in hand, a systematic investigation of the role
of polymer size and structure as well as polymer/DNA ratio on gene transfer properties was possible, in comparison to the ‘gold standard’ among polycationic transfection agents, namely the polycation PEI (both a linear and a branched PEI were included in the investigations). Compared to the commercial and presumably more heterogeneous PEI, the PDMAEMA gave very reproducible transfection results, already showing the need for defined polymers in the investigation of complex biological phenomena, such as genetic modification. Moreover, structure and N/P ratio were shown to have a significant impact on gene transfer, emphasizing the need for controlling and optimizing these parameters.

For both PEI and PDMAEMA, the linear molecules showed lowest transfection capability where that of the branched ones was significantly better; in our case the star-shaped PDMAEMA showed significantly better transfection capability, similar effect have been described for star-shaped PEI [29].

The cytotoxicity of the polycations alone in vitro conditions was in the following order: branched PEI > star-shaped PDMAEMA > linear PEI > linear PDMAEMA > highly-branched PDMAEMA. Cytotoxicity thus seems to correlate with transfection ability, since only the star-shaped PDMAEMA showed significant transfection capability. Whereas most previous researchers found that their polycations show much lower transfection efficiencies than PEI, the star-shaped PDMAEMA in our study was for certain N/P ratios as effective as PEI for transfecting CHO-K1 cells. Thus, the novel star-shaped PDMAEMA represents an efficient new gene delivery vector, which has the advantage of being more reproducible than PEI. In addition, it should be noted that the polyplexes based on star-shaped PDMAEMA are non-cytotoxic up to much higher N/P ratios than PEI-based ones. The ability of this vector to mediate transfection in other cell lines is under investigation.

Preliminary investigations of possible reasons for the differences in transfection efficiency observed for the different polycations show that all polycations ex cellular show a similar ability to compact DNA. Cellular uptake also seems to occur to a similar extent, independent of the structure and chemistry of the polycation. However, branched PEI-, star-shaped PDMAEMA- and to a lesser extent linear PEI-based polyplexes accumulate within sub-cellular structures as shown by the formation of spots of strong fluorescence within the cytoplasm. On the contrary, cells successfully transfected e.g. with linear PDMAEMA-based complexes display a fuzzy, less focussed pattern. Independently of the transfection outcomes, all tested polymers were detected only in the cytoplasm reinforcing the hypothesis that the critical step for successful transfection is the entry into the nucleus. Evidence for differences in cellular uptake or intracellular trafficking as basis for differences in transfection capability of the investigated polymers was not found.

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References