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Biomaterials 24 (2003) 1121–1131
Biomaterials
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In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis

Dagmar Fischer[a,*], Youxin Li[b], Barbara Ahlemeyer[c], Josef Krieglstein[c], Thomas Kissel[a]

[a] Department of Pharmaceutics and Biopharmacy, University of Marburg, Ketzerbach 63, 35032 Marburg, Germany
[b] Schwarz Pharma AG, Alfred-Nobel-Str. 10, 40789 Monheim, Germany
[c] Department of Pharmacology and Toxicology, University of Marburg, Ketzerbach 63, 35032 Marburg, Germany

Received 5 March 2002; accepted 9 September 2002

Abstract

A comparative in vitro cytotoxicity study with different water-soluble, cationic macromolecules which have been described as gene delivery systems was performed. Cytotoxicity in L929 mouse fibroblasts was monitored using the MTT assay and the release of the cytosolic enzyme lactate dehydrogenase (LDH). Microscopic observations were carried out as indicators for cell viability. Furthermore, hemolysis of erythrocytes was quantified spectrophotometrically. To determine the nature of cell death induced by the polycations, the nuclear morphology after DAPI staining and the inhibition of the toxic effects by the caspase inhibitor zVAD.fmk were investigated. All assays yielded comparable results and allowed the following ranking of the polymers with regard to cytotoxicity: Poly(ethylenimine) = poly(L-lysine) > poly(diallyl-dimethyl-ammonium chloride) > diethylaminoethyl-dextran > poly (vinyl pyridinium bromide) > Starburst dendrimer > cationized albumin > native albumin. The magnitude of the cytotoxic effects of all polymers were found to be time- and concentration dependent. The molecular weight as well as the cationic charge density of the polycations were confirmed as key parameters for the interaction with the cell membranes and consequently, the cell damage. Evaluating the nature of cell death induced by poly(ethylenimine), we did not detect any indication for apoptosis suggesting that the polymer induced a necrotic cell reaction. Cell nuclei retained their size, chromatin was homogenously distributed and cell membranes lost their integrity very rapidly at an early stage. Furthermore, the broad spectrum caspase inhibitor zVAD.fmk did not inhibit poly(ethylenimine)-induced cell damage. Insights into the structure–toxicity relationship are necessary to optimize the cytotoxicity and biocompatibility of non-viral gene delivery systems.

Keywords: In vitro cytotoxicity testing; L929 fibroblasts; Polycation; Hemolysis; Apoptosis

1. Introduction

Water-soluble polycations have been described for various technical and biomedical applications. Polymers such as poly(ethylenimine) (PEI) and poly (diallyl-dimethyl-ammonium chloride) (DADMAC) have been widely used for waste water treatment, separation of oil–water emulsions, as ionic retention aid, in shampoos and as flocculants in paper industry [1,2]. In biomedical applications they are under investigation as micro- and nanoparticulate drug carrier systems for proteins and peptides as well as non-viral vector systems for the transfer of DNA and RNA into cells and tissues [2–4]. Thus, these polymers need to be biocompatible, non-toxic, non-immunogenic, biodegradable, with a high drug-carrying capacity and controlled release of drugs at the target site [5]. The term "biocompatibility" encompasses many different properties of the materials, however, two important aspects of the biomaterial screening refers to their in vitro cytotoxicity and blood-compatibility behaviour [5].

In this study, we examined the in vitro cytotoxicity of a variety of water-soluble polycations considered as drug delivery systems especially for gene transfer. These polymers are used to form neutral or positively charged complexes with DNA due to electrostatic interactions [3]. Polycation/DNA complexes were usually found to be less cytotoxic than uncomplexed polycations [6]. Thus, our investigations reflects the worst case scenario for cytotoxicity. Most protocols use net cationic complexes with an excess of polymer to maintain an equilibrium between the complexed and dissociated form in solution. Furthermore, administration of the

*Corresponding author. Department of Pharmaceutics and Biopharmacy, Philipps-University, Ketzerbach 63, 35032 Marburg/Lahn, Germany. Tel.: +(49)-6421-282-5885; fax: +(49)-6421-282-7016.
E-mail address: fischerd@mailer.uni-marburg.de (D. Fischer).

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PII: S0142-9612(02)00445-3

polycation/drug complexes to blood can result in their dissociation and aggregation due to interactions with blood components such as erythrocytes and plasma proteins yielding non-complexed polycations. The free polymer then interacts with cell membranes, extracellular matrix proteins and blood components leading to undesired side effects [7].

Different techniques were used to measure changes in cell viability and interactions with erythrocytes. Membrane damage was quantified by the release of the cytosolic enzyme lactate dehydrogenase (LDH) since a potential site of interaction of cationic macromolecules is the cell membrane [8]. The LDH test provided information on the effects of polymers after short incubation times. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was selected to determine detrimental intracellular effects on mitochondria and metabolic activity. The colorimetric MTT test, based on the selective ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into purple formazan relies on intact metabolic activity and is frequently used for screening of cytotoxicity [9]. Changes in cell morphology and the detachment of cells from the petri dish were also used as indicators of cell survival by microscopic investigations. As target cells we used L929 mouse fibroblasts since they are recommended by many standard institutions [10,11] as reference cell line for the cytotoxicity testing of polymers. Furthermore, the blood compatibility of the polycations was quantified by spectrophotometric measurement of hemoglobin release from erythrocytes after polymer treatment. Finally, we studied the nature of cytotoxic reaction caused by polycations with respect to apoptotic or necrotic features.

2. Materials and methods

2.1. Polymers

Poly(diallyl-dimethyl-ammonium chloride) (DADMAC; 54 kDa) and poly(vinyl pyridinium bromide) (PVPBr; 17.9 kDa) were purchased from Polymer Standards Service GmbH (Mainz, Germany). Native human serum albumin (nHSA; fraction V; 67 kDa), diethylaminoethyl (DEAE)-dextran (500 kDa) and poly(L-lysine hydro bromide) (PLL; 36.6 kDa) were obtained from Sigma (Deisenhofen, Germany). Poly (ethylenimine) (PEI; 600–1000 kDa) was supplied by Fluka (Neu-Ulm, Germany). Starburst (PAMAM) dendrimer (generation 3, 20% (w/v) methanolic solution; 6909 Da) was purchased from Aldrich (Deisenhofen, Germany) and dried in vacuo before use. Cationized human serum albumin (cHSA; 67 kDa) was synthesized by covalent coupling of hexamethylenediamine to native human serum albumin and characterized as previously described [12]. Low molecular weight DEAE-dextrans were prepared by hydrolysis in water under reflux for 4 and 16 h at 100°C. The molecular mass was estimated measuring the inherent viscosity of the DEAE-dextrans. The determinations were carried out at 30°C using an Ubbelohde capillary viscosimeter (Schott Glas, Mainz, Germany) using 1% polymer solutions. Table 1 gives an overview over the characteristics of the cationic macromolecules.

2.2. Lactate dehydrogenase (LDH) assay

25,000 L929 mouse fibroblasts (DSMZ, Braunschweig, Germany)/cm^2 were cultured in six-well cell culture plates (Nunc, Wiesbaden, Germany) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Eggenstein, Germany) with 10% fetal calf serum (FCS, Gibco) and 2 mM glutamine at 37°C, 10% CO_2 and 95% relative humidity (r.h.). After 48 h cell culture medium was removed and replaced by 2 ml/well polymer solution. Hundred microlitre/well samples were collected after 0, 30 and 60 min of exposure. The LDH concentration in these samples was assayed utilizing a commercial kit (DG 1340-K, Sigma), allowing the spectrophotometric determination of the nicotinamide adenine dinucleotide (NAD) reduction at 340 nm in the presence of lactate and LDH according to the manufacturer's protocol. Controls were performed with 0.1% (w/v) Triton X-100

Table 1
Physicochemical properties of the polycationic macromolecules used in this study

Polymer	Molecular weight[a] (kDa)	Order of amines (°)	Charge/monomer ratio[b]	Structure
nHSA	67	1	n.d.	Globular-ellipsoid
cHSA	67	1	n.d.	Globular
PAMAM	6.9	1, 3	0.0088	Star-fish-shaped
PVPBr	17.9	4	0.0054	Linear, aromatic
DEAE-dextran	500	3	0.00278	Linear, intramolecular crosslinking
DADMAC	54	4	0.00619	Linear, flexible
PLL	36.6	1	0.00685	Linear, flexible
PEI	600–1000	1, 2, 3	0.0233	Branched

[a] According to the manufacturers' information.
[b] Determined as the number of cationic charges per molecular weight of the polymeric monomer.

and set as 100% LDH release. The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells. Less than 10% LDH release were regarded as non-toxic effect level in our experiments. All samples were run in triplicate and experiments were repeated twice.

2.3. MTT assay

The MTT assay was performed according to the method of Mosmann [9] and Zange [13]. All experiments were run eight times. Polymer solutions were prepared in serum supplemented tissue culture medium and sterilized by filtration (0.2 μm, Schleicher & Schuell, Dassel, Germany). To exclude cytotoxic effects due to changes in osmolarity and pH of the polymer solutions compared to cell culture medium (pH 7.4, 295 mosm/kg), physiologically well tolerated values of pH 7.4 and 280–320 mosm/kg were adjusted. L929 cells were plated into 96-well microtiter plates (Nunc) at a density of 25,000 cells/cm^2. After 24 h, culture medium was replaced by 100 μl serial dilutions of the polymers and the cells were incubated for 3, 12, and 24 h. Polymer solutions were aspirated and replaced by 200 μl DMEM without serum. Twenty microlitres sterile filtered (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT, Sigma) stock solution in phosphate buffered saline (PBS) pH 7.4 (5 mg/ml) were added to each well reaching a final concentration of 0.5 mg MTT/ml. After 4 h unreacted dye was removed by aspiration, the insoluble formazan crystals were dissolved in 200 μl/well dimethylsulfoxide (Merck, Darmstadt, Germany) and measured spectrophotometrically in an ELISA reader (Titertek Plus MS 212, ICN, Eschwege, Germany) at a wavelength of 570 nm (test) and 690 nm (reference). The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without polymer was calculated by [A] test/[A] control $\times$ 100. The IC_{50} was calculated as polymer concentration which inhibits growth of 50% of cells relative to non-treated control cells.

2.4. Microscopic observations

After incubation with polymers, changes in morphology and detachment of L929 fibroblasts from the dish were observed using a Nikon inverse phase contrast microscope (Nikon TMS, Nikon, Japan) equipped with an objective (Plan 10/0.30DL/Ph1, Nikon, Japan) of $\times$ 100 magnification.

2.5. Hemolysis test

The hemolytic activity of the polymers was investigated according to Parnham and Wetzig [14]. Blood, collected in heparinized-tubes from Wistar rats, was centrifuged at 700g for 10 min. The pellet was washed three times with cold PBS pH 7.4 by centrifugation at 700g for 10 min and resuspended in the same buffer. This suspension of red blood cells was always freshly prepared and used within 24 h after collection. Polymer solutions of different concentrations, also prepared in the PBS buffer, were added to the erythrocytes and were incubated for 60 min at 37°C in a shaking water bath. The release of hemoglobin was determined after centrifugation (700g for 10 min) by photometric analysis of the supernatant at 540 nm. Complete hemolysis was achieved using 0.2% Triton X-100 yielding the 100% control value. Less than 10% hemolysis were regarded as non-toxic effect level in our experiments. The experiments were run in triplicate and were repeated twice.

2.6. Characterization of polymer-induced cell death

To evaluate whether cell death induced by the polymers has apoptotic or necrotic features, SW 13 adrenal gland carcinoma cells (kind gift of Dr. Aigner, Department of Pharmacology and Toxicology, Marburg), which were grown in RPMI1640 (Gibco) supplemented with 10% FCS and 2 mM glutamine at 37°C, 10% CO_2 and 95% r.h., were treated with 800 kDa PEI/DNA complexes (0.5 μg DNA/10,000 cells), equivalent concentrations of 800 kDa PEI (0.06 mg/ml) without DNA and 200 nM staurosporine (Sigma) for 4 h in the presence and absence of 100 μM broad spectrum caspase inhibitor zVAD.fmk (Bachem, Heidelberg, Germany). All complexes were prepared at a nitrogen/phosphate (N/P) ratio of 40 using herring testes DNA (Sigma) as described earlier [15]. Staurosporine, a selective inducer of apoptosis [16] was used as positive control by comparing its effects on nuclear morphology, LDH release and metabolic activity with that of the polymers and the polymer/DNA complexes. Caspase-3 activation is considered as a hallmark of apoptosis, and thus apoptosis, but not necrosis can be blocked by caspase inhibitors. After treatment, a part of the cultures was stained with 4′, 6′-diamidino-2-phenylindole (DAPI, Sigma) to visualize nuclear morphology. Briefly, cultures were stained for 10 min with 150 ng/ml DAPI, washed with methanol and PBS and were viewed under a confocal laser scanning microscope (LSM 510, Zeiss, Jena, Germany) at 364 nm. Two other parts of the cultures were used for LDH and MTT assay which were performed as described above.

2.7. Statistics

Significant differences between two groups were evaluated by Student's t-test and between more than

two groups by one-way analysis of variance followed by Scheffé-test.

3. Results

3.1. Polymers induced substantial morphological changes in L929 mouse fibroblasts

Depending on the type of polycation, substantial changes in cell morphology were already detected microscopically 1 or 3 h after exposure with the respective polymer, becoming more prominent during longer incubation periods. Usually, L929 mouse fibroblasts are large, spindle-shaped, adherent cells growing as a confluent monolayer (Fig. 1a). Using PEI as an example, at 0.001 mg/ml no or little damage could be detected compared with control cells (Fig. 1b). Higher polymer concentrations and longer incubation times generated more pronounced cell debris and changes in morphology, such as cell lysis, loss of spindle shape and sometimes detachment from the bottom (Fig. 1c, d).

3.2. Polymers caused membrane damage in L929 mouse fibroblasts detected by LDH assay

To assess the damage of cell membranes, as the initial point of interaction with polycations, the extracellular concentration of LDH was quantified. Three different concentrations (0.01, 0.1 and 1 mg/ml) were tested over 0, 30 and 60 min exposure (Fig. 2). The 0 min value was chosen to confirm the viability of the cells. Under these conditions nHSA, cHSA, PAMAM, DEAE-dextran and PVPBr did not induce significant LDH release ($< 5\%$). However, PLL, PEI and DADMAC caused a significant membrane damage after 60 min already at

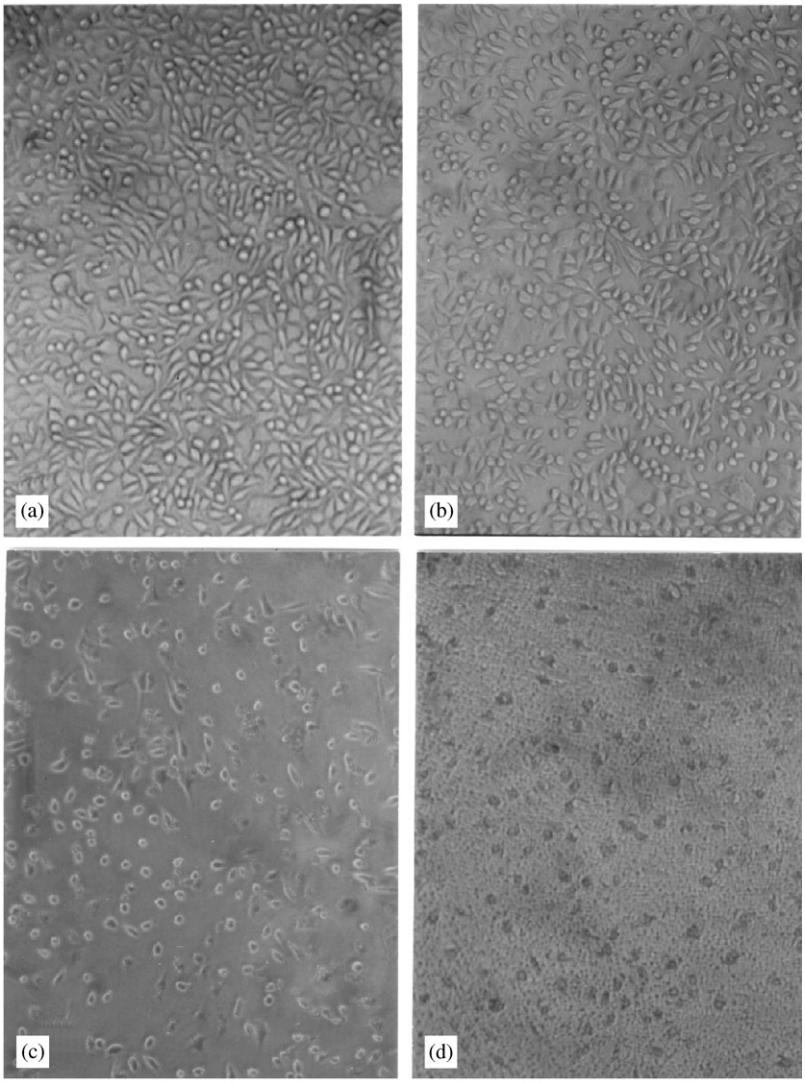


Fig. 1. Morphology of the L929 cells before and after exposure to PEI for 3 h. (a) Control cells were compared with cells incubated with (b) 0.001 mg/ml PEI, (c) 0.01 mg/ml and (d) 0.1 mg/ml PEI (100 ×).

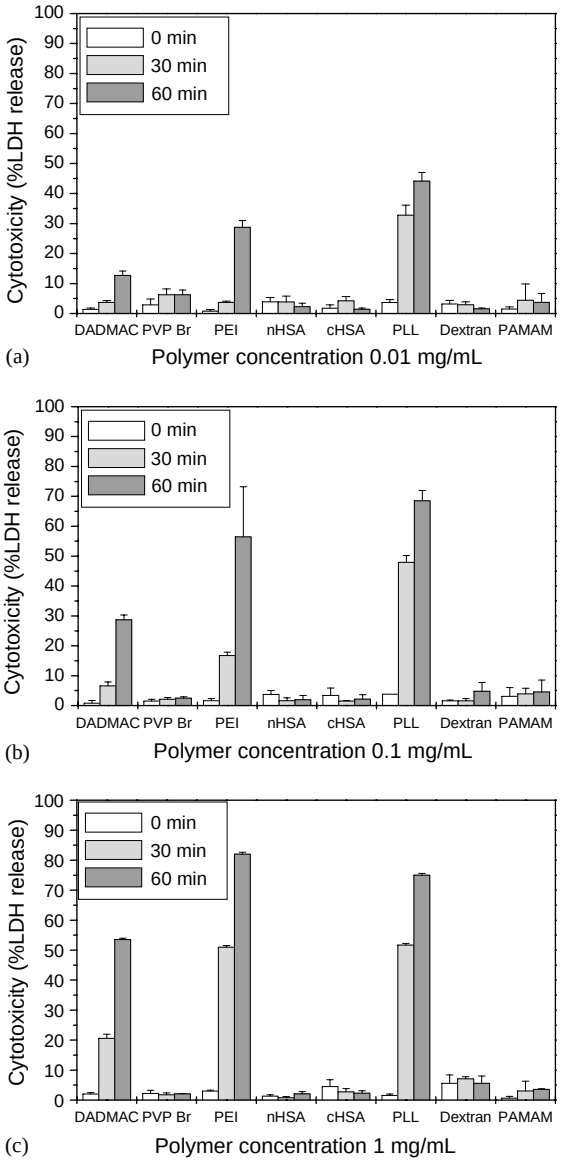


Fig. 2. Dose- and time-dependent effects of different polycationic macromolecules on L929 cells after 0, 30 and 60 min. The membrane damaging effect of three polymer concentrations (a) 0.01 mg/ml, (b) 0.1 mg/ml and (c) 1 mg/ml was quantitated by the release of the cytosolic enzyme LDH. Each value represents the mean ± SD of three determinations.

0.01 mg/ml which increased with higher polymer concentrations in the order of PLL ≥ PEI > DADMAC. In control cells which were not treated with polymer, the release of the enzyme was found to be in the range from 1 to 3% (0 min: 0.96 ± 0.36 mg/ml, 30 min: 1.27 ± 0.41 mg/ml, 60 min: 0.87 ± 0.64 mg/ml).

3.3. Polymers induced a decrease of metabolic activity in L929 mouse fibroblasts

To determine effects of the polymers on metabolic activity the MTT test was used. All polymers affected the metabolic activity in a concentration- and time-dependent manner when they were added in the concentration range 0–10 mg/ml for up to 24 h to the cells. Cytotoxicity of the cationic polymers decreased in relation to decreasing concentrations and incubation times as summarized in Table 2 showing the IC_{50} values of the polycations tested at 3, 12 and 24 h.

Under these conditions nHSA and cHSA revealed no or only moderate cytotoxic effects up to 10 mg/ml (Fig. 3). nHSA concentrations ≥ 1 mg/ml increased the viability of the cells about 15-50% dependent on the exposure time. The increased cell viability with native albumin can be explained by a nutrient effect. Incubated with cHSA for 12 h and 24 h, the fibroblasts showed significant loss in viability of about 37–50% observed at concentrations ≥ 5 mg/ml. Below this concentration, cellular metabolic activity did not change in comparison with control cells.

After 3 h, we determined more than 80% viable cells after incubation with all polymers at 0.01 mg/ml with the exception of PLL (68.3%). After 12 h of exposure to 0.01 mg/ml PVPBr, PAMAM and DADMAC all cells were viable, whereas PLL (73.2%) and PEI (72.3%) showed a slight and cationic dextran (44.6%) a significant decreased cell growth. After 24 h only DEAE-dextran (30.5%) and PEI (47.0%) inhibited the metabolic activity, all other polycations showed moderate toxic effects (Fig. 4a). Independent from the time of exposure, 0.1 mg/ml PEI and PLL caused a complete loss of cell viability. Incubation with 0.1 mg/ml DADMAC and DEAE-dextran, which caused severe cell death after 12 and 24 h, lowered the metabolic activity to 29.6% and 72.5% after 3 h, respectively. PVPBr and PAMAM induced only a moderate decrease in cell viability and are considered as polycations with a good compatibility (Fig. 4b). No cells survived exposed to PLL, PEI and DADMAC (1 mg/ml) for 3, 12 and 24 h. Whereas more than 50% viable cells could be detected after 3 h in cultures treated with 1 mg/ml DEAE-dextran (55.6%) and PVPBr (64.7%), after 12 h and 24 h cell

Table 2
IC_{50} of different polycationic macromolecules. L929 mouse fibroblasts were incubated for 3, 12 and 24 h with different concentrations of polymer solution. Cell viability was quantified by MTT assay as described in Materials and Methods ($n = 8$)

Polymer	3 h	12 h	24 h
nHSA (mg/ml)	> 10	> 10	> 10
cHSA (mg/ml)	> 10	> 10	9.28
PAMAM (mg/ml)	> 10	> 10	> 10
PEI (mg/ml)	0.031	0.022	0.009
PLL (mg/ml)	0.032	0.040	0.038
DADMAC (mg/ml)	0.096	0.048	0.034
PVPBr (mg/ml)	1.45	0.492	0.246
DEAE-dextran (mg/ml)	> 2	0.011	0.011

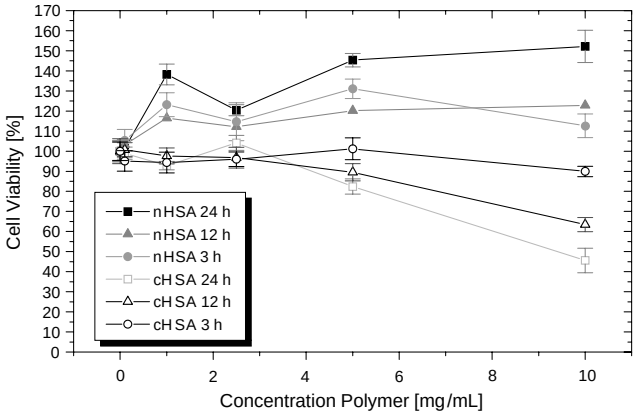


Fig. 3. Effect of nHSA and cHSA on L929 cell viability measured by MTT assay after 3, 12 and 24 h incubation at 37°C. Each point represents the mean ± SD of eight determinations.

viability was completely inhibited. At 1 mg/ml for PAMAM only a low extent of toxicity could be detected even after 24 h (Fig. 4c).

3.4. Cytotoxicity of the polycations is depending on their molecular weight

Commercially available 500 kDa DEAE-dextran was hydrolyzed over 4 and 16 h to products with lower molecular weights, which were characterized by capillary viscosimetry. Fig. 5a summarizes the characteristics of the DEAE-dextrans, their inherent viscosities and the time of hydrolysis. The influence of the three cationic dextrans on the metabolic activity of the fibroblasts was in the same range after 3 h incubation time. Significant differences between the high and the low molecular weight polymers could be observed 12 and 24 h after exposure. As shown in Fig. 5b, incubation with high molecular weight 500 kDa DEAE-dextran (1 mg/ml) for 12 and 24 h showed drastic cytotoxic effects in L929 cells. After 3 h, viability was reduced to 55.6%. In comparison, the low molecular weight dextrans significantly influenced cell viability only after 24 h exposure time reducing the number of cells to 14.0% (5.647) and 32.3% (4.199). After 3 and 12 h of incubation 60–70% and 50–60% of the cells were viable and metabolically active for dextran 4.199 and dextran 5.647, respectively. Concentrations in the range 0.001–0.1 mg/ml, which were also tested by the MTT-assay (data not shown), gave similar results.

3.5. Determination of the hemolytic activity

The interactions of the cationic polymers with negatively charged membranes have also be studied by hemolysis experiments. The release of hemoglobin was used to quantify the membrane-damaging properties of the polymers. As 100% and 0% values we used Triton

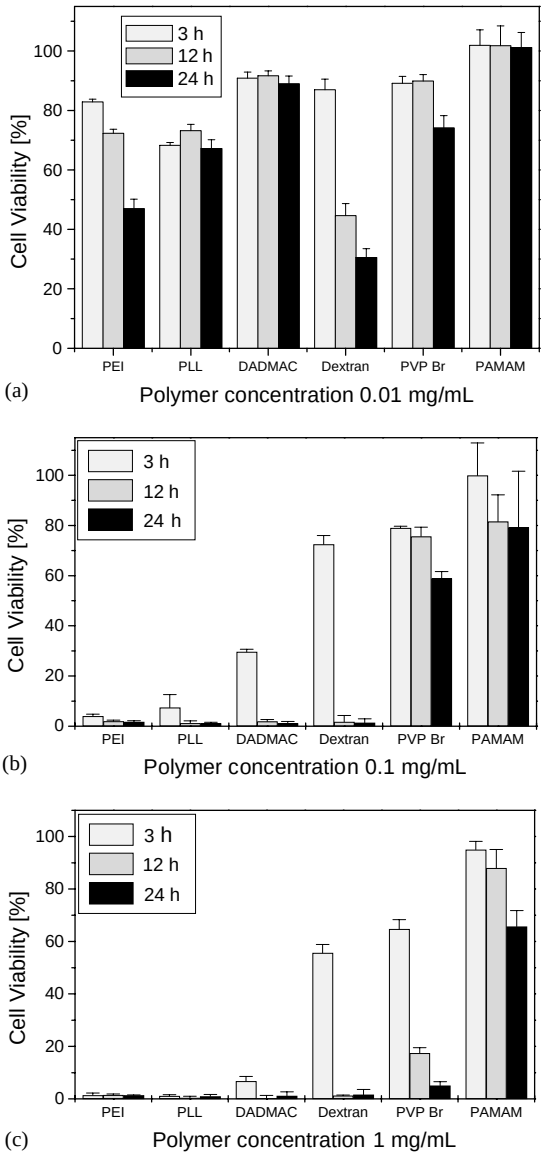


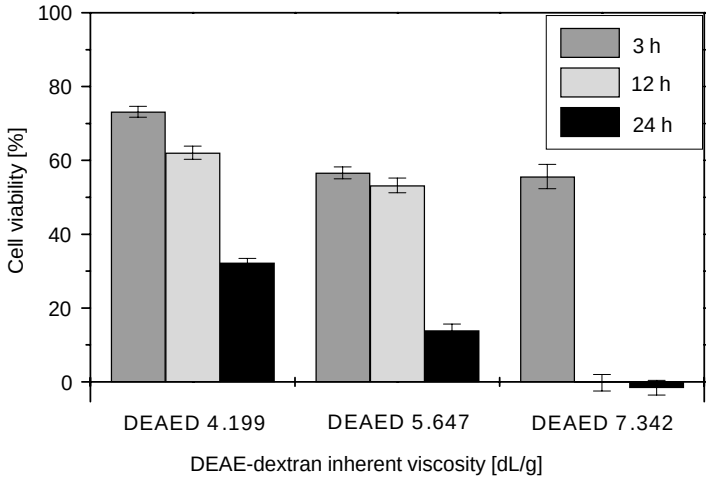
Fig. 4. Cytotoxic effects of different polycations on L929 fibroblasts after 3, 12 and 24 h incubation time using (a) 0.01 mg/ml, (b) 0.1 mg/ml and (c) 1 mg/ml polymer solution. The cell viability was determined by MTT assay and was shown as mean ± SD of eight determinations.

X-100 and phosphate-buffer-treated erythrocytes, respectively. Erythrocytes were incubated with five different polymer concentrations in the range of 0.01–10 mg/ml for 1 h (Table 3). Under these conditions, nHSA, cHSA, PAMAM, PVPBr and DEAE-dextran showed no hemolytic effects up to 10 mg/ml indicating no detectable disturbance of the red blood cell membranes. PLL and DADMAC (10 mg/ml) caused 13.59% and 24.51% hemolysis, respectively. PEI was found to be the most membrane-damaging polymer reaching complete lysis of the erythrocytes at concentrations $\geqslant$ 1 mg/ml. Consistent with the LDH assay, PEI, PLL and

DEAD-dextran	Time of hydrolysis	Inherent viscosity
DEAE-dextran HMW	---	7.342 dL/g
DEAE-dextran MMW	4 h	5.647 dL/g
DEAE-dextran LMW	16 h	4.199 dL/g

HMW high molecular weight
MMW medium molecular weight
LMW low molecular weight

(a)



(b)

Fig. 5. Decrease of cell viability as a function of molecular weight. (a) Characteristics of the DEAE-dextrans. (b) Mouse fibroblasts were incubated with the three DEAE-dextrans (1 mg/ml) of different molecular weights for 3, 12 and 24 h. Cell viability was measured by MTT assay ($n=8$).

Table 3
Percentage release of hemoglobin (mean ± SD) of rat red blood cells after 60 min incubation with different concentrations of the cationic polymers at 37°C ($n=3$)

Polymer	0.01 mg/ml	0.1 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml
nHSA	1.56 ± 2.87	2.25 ± 1.04	6.94 ± 0.97	7.38 ± 0.29	7.07 ± 0.29
cHSA	4.21 ± 3.48	0.91 ± 2.19	7.09 ± 0.64	7.30 ± 0.24	6.25 ± 0.12
PAMAM	2.95 ± 0.92	7.40 ± 2.56	3.72 ± 0.80	7.93 ± 3.84	0.84 ± 0.92
DEAE-dextran	2.75 ± 0.50	1.23 ± 0.88	5.50 ± 1.75	1.70 ± 0.18	2.96 ± 1.84
PLL	7.88 ± 2.71	10.27 ± 4.52	6.44 ± 2.73	8.75 ± 1.97	13.59 ± 0.76
DADMAC	3.90 ± 0.21	3.33 ± 1.39	2.60 ± 0.38	2.38 ± 1.42	24.51 ± 1.32
PEI	6.09 ± 1.60	9.02 ± 0.10	92.67 ± 1.34	87.58 ± 0.30	92.87 ± 0.85
PVPBr	5.21 ± 1.52	5.71 ± 0.82	9.42 ± 1.77	10.12 ± 2.72	9.55 ± 2.34

DADMAC again were found to be highly membrane-interrupting polymers.

3.6. Characterization of the nature of cell death induced by PEI and PEI/DNA complexes

To characterize the cytotoxic effects induced by the polymers with respect to apoptosis and necrosis, SW13 cells were employed since L929 fibroblasts responded only weakly to staurosporine. As mentioned above, SW13 cells were treated with the DNA/polymer complexes as well as the uncomplexed polymer, but also with staurosporine as positive control and with the broad spectrum caspase inhibitor zVAD.fmk which blocks caspase-dependent apoptotic, but not necrotic death. Caspases are the main executioners of the apoptotic cell death. They recognize a very short tetrapeptide sequence within targed substrate polypeptides and these motifs have formed the basis for inhibitors. Irreversible caspase inhibitors are of the general structure [tetrapeptide]–$CO–CH_2–X$ including ketones, where X is –Cl or –F (chloro- or fluoroketone). zVAD.fmk is therefore the abbreviation of a fluoroketone = fmk and VAD is the abbreviation of the tetrapeptide [17].

Apoptosis can be detected by nuclear morphology after staining with DAPI. Vehicle-treated SW13 cells contained round nuclei with homogeneous chromatin (Fig. 6a), whereas cells exposed to staurosporine for 4 h

showed a reduction in nuclear size, chromatin condensation as well as nuclear fragmentation as typical features of apoptosis (Fig. 6b) which was completely blocked in the presence of the caspase inhibitor zVAD.fmk (Fig. 6f). Normal nuclear morphology was observed in cultures treated with PEI (Fig. 6c,g) and PEI/DNA complexes (Fig. 6d,h) in the presence as well as in the absence of zVAD.fmk. Furthermore, morphology of SW13 cells treated with the vehicles was checked by phase contrast microscopy. As already described for L929 fibroblasts, also SW13 cells were found to be dramatically damaged after the treatment with PEI and PEI/DNA complexes in the absence as well as in the presence of the caspase inhibitor zVAD.fmk (data not shown). Thus, cell death caused by PEI and PEI/DNA complexes did not display the typical nuclear changes occuring during apoptosis. Membrane damage which can be determined by LDH assay, is known to occur only in the late stage of apoptosis. LDH release was only weakly detectable in staurosporine-treated SW13 cells, but was dramatically increased in SW13 cells 4 h after addition of PEI and PEI/DNA complexes (Fig. 6i). The data confirm the notion of a necrotic cell damage induced by polymers. Mitochondrial dysfunction is another key event in apoptosis which can be determined by MTT assay, although this method does not allow

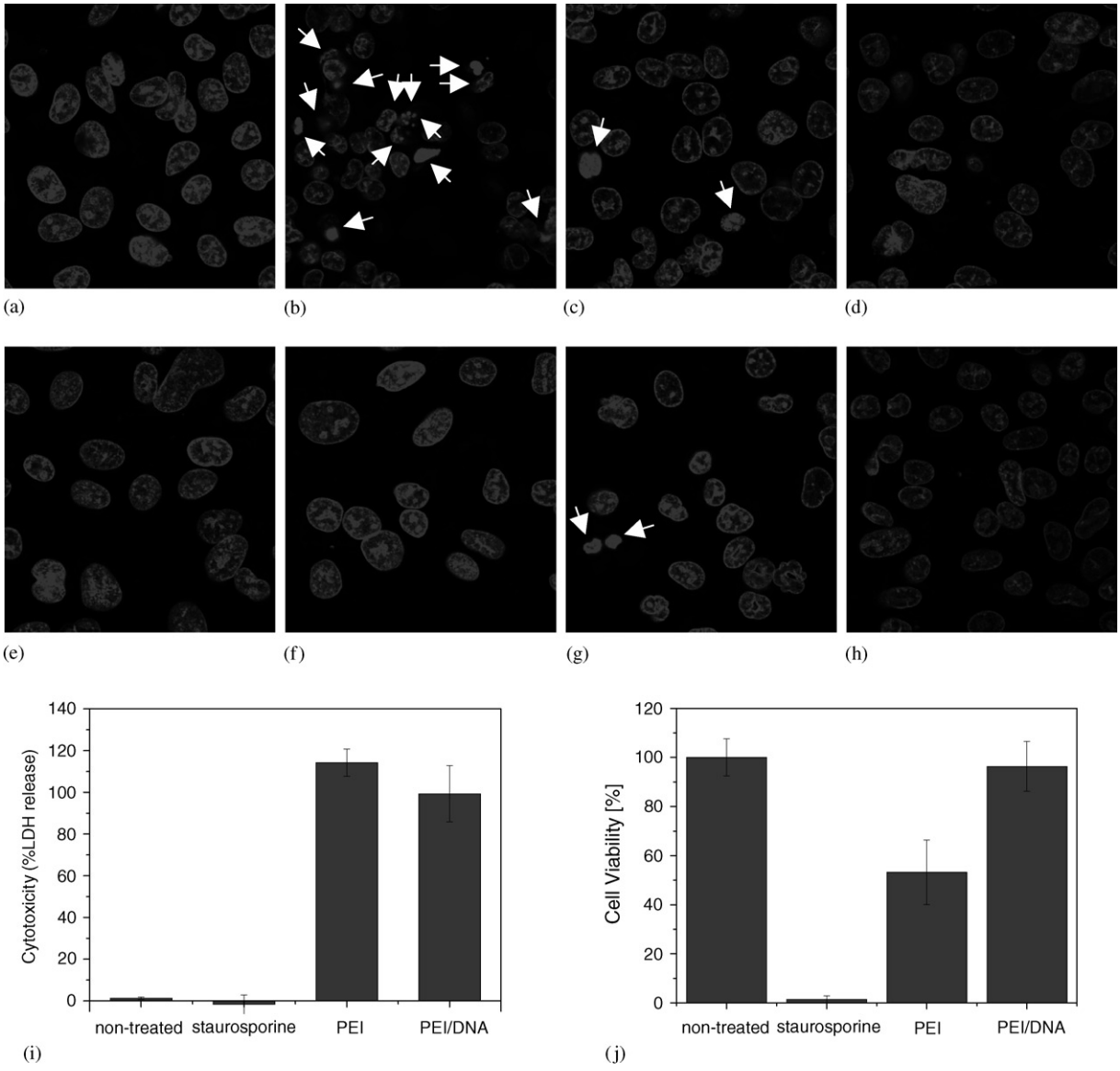


Fig. 6. Staurosporine, but not PEI and PEI/DNA complexes-induced apoptosis in SW13 carcinoma cells. Photomicrographs of the cells treated for 4 h with vehicle (a,e), 200 nM staurosporine (b,f), PEI (c,g) and PEI/DNA (N/P 40) complex (d,h) in the absence (a–d) or presence (e–h) of 100 μM zVAD.fmk. Cells were stained with DAPI to visualize nuclear morphology. Please note the higher number of cells with chromatin condensation, reduction of nuclear size as well as nuclear fragmentation (indicated with white arrows) in staurosporine—than in vehicle—, PEI- or PEI/DNA complex-treated cultures. The broad spectrum caspase-inhibitor zVAD.fmk reduced apoptotic damage induced by staurosporine (b,f). For LDH (i) and MTT (j) assay SW13 cells were treated for 4 h as described in Materials and Methods.

crimination between apoptosis and necrosis. Whereas staurosporine treatment reduced the metabolic activity to 0%, PEI/DNA complexes as well as the equivalent amount of non-complexed PEI induced less disturbance of the metabolic activity of the cells (Fig. 6j).

4. Discussion

The cytotoxicity of polycationic macromolecules with different structures was investigated under in vitro conditions. Based on our results, the cytotoxicity can be ranked as follows: PEI = PLL > DADMAC > DEAE-dextran > PVPBr > PAMAM > cHSA > nHSA.

Although different assays and cell types were used, the results and the rank order of the polymers with regard to their cytotoxicity were in a comparable range. MTT and LDH assay gave similar results, since membrane damage after 1 h (LDH) was always followed by a decrease of the metabolic activity (MTT) 3 h after exposure to the polymers. The severe membrane damage caused by PEI, PLL and DADMAC at 1 h resulted at 3 h in a loss of the metabolic activity. For DEAE-dextran, PAMAM and PVPBr which do not induce measurable LDH release after 60 min, also no significant decrease of metabolic activity could be observed at 3 h. Similary, exposure to nHSA and cHSA did not change the release of LDH or metabolic activity demonstrating their good cell compatibility. Differences between the membrane destructive effects of the polymers on L929 mouse fibroblasts and red blood cells might be due to the different composition of the membranes and glycocalyx of fibroblasts and erythrocytes.

Biocompatibility is thought to be influenced by different properties of the polymers such as (i) molecular weight, (ii) charge density and type of the cationic functionalities, (iii) structure and sequence (block, random, linear, branched) and (iv) conformational flexibility [5,8,18]. An increase in cytotoxicity as a function of the molecular weight, which was observed for DEAE-dextran in this study, was also reported for other polycations, e.g. PLL [18,19], dendrimers [20] and PEI [15]. However, these findings apply only for polymers from same structure, but not for different types of polycations. For example, in our experiments PLL with 36.6 kDa influenced cell viability even more than cHSA with 67 kDa and the 500 kDa DEAE-dextran. Consequently, to explain the toxicity of polymers with different structures further parameters have to be taken into account.

Different authors discussed the role of the type of amines for toxicity. Dekie et al. [21] concluded from their experiments with poly L-glutamic acid derivatives that the presence of primary amines has a significant toxic effect on red blood cells causing them to agglutinate. Based on studies with modified PLL, Ferruti et al. [22] noted that macromolecules with tertiary amine groups exhibit a lower toxicity than those with primary and secondary residues. Our study confirms these observations for PLL and PEI, by contrast, cHSA and PAMAM which also contain primary amino groups, showed only moderate cytotoxic effects.

Therefore, not only the type of amino function but also the charge density resulting from the number and the three-dimensional arrangement of the cationic residues is an important factor for cytotoxicity. Ryser [23] suggested a three-point attachment to be necessary for eliciting a biological response on cell membranes and speculated that the activity of a polymer will decrease when the space between reactive amine groups is increased in the primary structure. The arrangement of cationic charges depends on the three-dimensional structure and flexibility of the macromolecules [8,23] and determines the accessibility of their charges to the cell surface. Branched molecules were found to be more efficient in neutralizing the cell surface charge than polymers with linear or globular structures [8]. Rigid molecules have more difficulties to attach to the membranes than flexible molecules [23,24]. Therefore, high cationic charge densities and highly flexible polymers should cause higher cytotoxic effects than those with low cationic charge densities. This hypothesis was confirmed by our experiments. The polycations characterized by a globular structure (cHSA, PAMAM) were found to be the polymers with good biocompatibility, whereas polymers with a more linear or branched and flexible structure (DADMAC, PLL, PEI) showed higher cell damaging effects. As a trend, an increase of the charge/monomer ratio, determined as the number of cationic charges per monomer unit, is correlated with an increase in the cytotoxic effects (Table 1).

PEI was found to be the polymer with the highest cytotoxicity in our study. This observation is correlated with the large molecular size as well as the high number of charges. Due to the high degree of branching, PEI has a higher charge density compared to equally sized polycations with a linear structure. Although both, PLL and DADMAC are linear, flexible polymers of comparable molecular weights and charge/monomer ratios, they showed differences in their cytotoxicity. The toxicity profile of PLL in our study is in good agreement with those reported by others [5,8]. Based on electron spin resonance (ESR) measurements, Hartmann et al. [25] suggested that in contact with biological membranes the transformation of the randomly coiled PLL changes to an α-helix in such a way that a maximum number of lysine groups can bind to the membrane surface. Half of the lysine groups are oriented to the membrane glycocalyx while the other half is extended into the surrounding water phase. The lower toxicity of DADMAC compared to PLL may be presumably due to the

ring systems of the DADMAC making the polymer more stiff and rendering the attachment of DADMAC to the cell surface more difficult than using PLL. Although PVPBr also contains quarternary amino groups, but a higher charge/monomer ratio compared to DADMAC, it showed a more favourable cytotoxicity profile, presumably due to delocalization of the positive charge over the aromatic ring system. Similar data were reported by Kabanov et al. [26] studying the cytotoxicity of quaternized polyvinyl pyridinium derivatives on 3T3 fibroblasts.

With regard to the charge/monomer ratio PAMAM and DEAE-dextran were found to differ significantly. The low cytotoxicity of the PAMAM inspite of a high charge monomer ratio can be explained by the lower molecular weight compared with the other polycations. Additionally, under physiological conditions, the polymer is only partly protonated [20]. Although DEAE-dextran has a high molecular weight, number and density of the cationic charges are low, explaining the good compatibility. Furthermore, intramolecular cross-linking in the molecules led to a rigid behaviour, prevented strong binding to the cell membrane and reduced cell viability significantly only at higher polymer concentrations [23]. The low toxicity of the native albumin is due to the net negative charge under physiological conditions which distinguish it from all other polymers. For the good biocompatibility of the cHSA, which was also reported by others [8], its low charge density as well as the globular structure were assumed to be responsible.

The mechanism of cytotoxicity caused by polycations is not yet fully understood. Whether cytotoxic effects are mainly mediated by interactions of polycations with cell membranes or by cellular uptake and subsequent activation of intracellular signal transduction pathways, is still discussed controversially in the literature. Furthermore, no investigations were reported to elucidate the apoptotic or necrotic nature of the polycations' cytotoxicity.

Initial interaction between cationic macromolecules and the negatively charged cell membrane is mediated by electrostatic interactions [8]. Interactions with membrane proteins and phospholipids seem to disturb membrane structure and function [18,19]. Arnold et al. [27] suggested that poly(L-lysine) triggers cellular efflux of organic and inorganic substances proportional to its membrane adsorption. Malik et al. [28] demonstrated the membrane interactions of polycations causing erythrocyte lysis. The participation of the protein kinase casein kinase II (CK II) was discussed, based on observations that in vitro CK II was markedly activated by polycationic structures such as polyamine and spermine [29]. The cytotoxicity of poly(L-lysine) was not influenced by inhibition of microtubules and microfilament formation indicating that the internalization of the polymer was not a prerequisite for the observed cytotoxic effects [19].

From the results of LDH and MTT assay, we conclude that in cells exposed to cationic polymers first membrane leakage occurred followed by a decrease in metabolic activity. In preliminary experiments [30], confocal laser scanning microscopy revealed disruption of the plasma and nuclear membrane after a 60 min exposure of 800 kDa PEI which was assumed to accomplish the direct penetration of the polymer through the cell membrane into the cytoplasm and nucleus, respectively. Also interactions with cytoskeleton elements could be visualized.

Since the early and rapid loss of plasma membrane integrity suggests a necrotic type of cell death, we have performed additional experiments to obtain further insight about the nature of cell death and to differentiate between apoptosis and necrosis. The common methods to characterize apoptosis are the demonstration of morphological changes such as shrinkage and fragmentation of the nucleus and the DNA which can be investigated by nuclear staining with Hoechst, TUNEL-staining and DNA gel electrophoresis as well as biochemical changes such as the activation of caspases. In this study, we used nuclear staining with Hoechst, because false positive results have been reported for the TUNEL assay. The demonstration of DNA laddering by DNA electrophoresis often fail because of a too low and thus invisible amount of DNA in cultured cells. Activation of caspases was indirectly proved by the efficiency of the caspase inhibitor. As a positive control, cells were treated with staurosporine, a selective inducer of apoptosis [16]. Staurosporine-induced death showed typical features of apoptosis such as reduction in nuclear size, chromatin condensation and nuclear fragmentation and cell death was inhibited by caspase inhibitor, whereas the plasma membrane remained intact. In contrast, cell death induced by PEI and its DNA complexes showed features of necrosis as evidenced by an early membrane leakage without changes in nuclear morphology. In addition, PEI and PEI/DNA complex-induced damage was not inhibited by the caspase inhibitor zVAD.fmk, a further indication for a necrotic type of cell death. Furthermore, the suggested destruction of lysosomes induced by PEI by proton sponge hypothesis is a phenomenon typically observed during necrosis.

In conclusion, the cytotoxicity of cationic polymers with different structures was investigated. Different assays, quantifying the influence of the polycations on the membrane integrity and the metabolic activity of the cells, gave similar results even for different cell types. Whereas the molecular weight correlated with the toxicity of polymers with the same monomeric structure, the cell damaging effects of different types of polycationic macromolecules were found to depend on the

number and arrangement of the cationic charges which determine the degree of interaction with the cell membranes. Furthermore, characterization of cell death induced by PEI and PEI/DNA complexes suggests a necrotic type of death. These insights into the structure–toxicity relationship could be helpful for optimizing the biocompatibility of polymeric delivery systems.

Acknowledgements

We would like to thank Markus Burkert for his excellent technical assistance.

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