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# Polymer Complexes in Biological Applications

**Jana Hedrich, Yuzhou Wu, Seah Ling Kuan, Frauke Kuehn, Eweline Pietrowski, Mike Sahl, Sandra Muth, Klaus Müllen, Heiko J. Luhmann, Tanja Weil, and Manfred Schmidt**

**Abstract** This chapter summarizes the influence of polyelectrolyte topology on biological functions and biomedical applications such as cell uptake, drug delivery, and gene transfection. Polyelectrolytes utilized are spherical structures derived from dendrimers and albumin or cylindrical brushes, all of which are decorated with various polypeptide chains.

First, experiments are described that address the role of polyelectrolyte interactions on endosomal uptake and release, followed by a discussion of the novel design of albumin-based nanocarriers for anticancer drugs like doxorubicin. Finally, we describe how efficient gene transfection was accomplished with both albumin-based polycations and with cylindrical brushes having poly-L-lysine side chains.

**Keywords** Brain • Cylindrical brush polymer • Dendritic and protein polyelectrolytes • Endothelial cell • Flow cytometry • Gene transfer • Polycation • Transfection

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## Abbreviations

AML	Acute myeloid leukemia
ATRP	Atom transfer radical polymerization
cBSA	Cationized bovine serum albumin
DHSA	Dendronized human serum albumin
DMF	Dimethyl formamide
DOX	Doxorubicin
ECM	Extracellular matrix
EPR	Enhanced retention and permeation
GFP	Green fluorescent protein
HSA	Human serum albumin
LiBr	Lithium bromide
MRI	Magnetic resonance imaging
$M_w$	Weight-average molecular weight
NaCl	Sodium chloride
PAMAM	Polyamidoamine
PBMEC	Porcine microvascular endothelial cells
PDI	Perylene-3,4,9,10-tetracarboxdiimide
PEG	Poly(ethylene glycol)
PEI	Poly(ethylene imine)
PEO	Poly(ethylene oxide)
PLL	Poly-L-lysine
$P_n$	Number-average degree of polymerization
$P_w$	Weight-average degree of polymerization
$R_g$	Radius of gyration
$R_h$	Hydrodynamic radius

TEM	Transmission electron microscopy
$w_{\text{DNA}}$	Weight fraction of DNA
$z^+/z^-$	Molar charge ratio polycation/polyanion

# 1 Exploring Cell Interactions of Dendritic and Protein Polyelectrolytes

## 1.1 Introduction

Ionic interactions play a crucial role in many cellular processes such as membrane permeation or regulation of gene transcription [1]. The cell membrane, with its bilayer and negatively charged surface, serves as a protective barrier for the living cell towards its environment. Hydrophobic or positively charged molecules can interact with the cell membrane and can enter cells via various mechanisms [2]. In the cell nucleus, polycationic proteins and histones form complexes with DNA and regulate transcription through alteration of the charge densities of nuclear proteins and DNA [3]. Inspired by these polyelectrolytic interactions in nature, synthetic nanosized polyelectrolytes have been designed that contain multiple electrolyte groups, i.e., polycations or polyanions. Some of these polyelectrolyte carriers have already been successfully explored for biomedical applications such as gene or cancer therapy [4, 5]. With greater emphasis on defined macromolecular architectures of the materials, regularly branched cascade molecules (so-called dendrimers) and protein-based polyelectrolytes have emerged over the past decade. Their interactions with cells have been analyzed to elucidate the role of polyelectrolyte interactions in nature and to apply this knowledge for the design of efficient and biocompatible nanotransporters for the delivery of cargoes such as drugs, proteins, or nucleic acids into cells and subcellular compartments [6, 7].

Endocytosis is essential for uptake of macromolecules and nanoparticles. Thereby, the physical and chemical characteristics of the cargo and transporter complex determine the nature of the cell uptake mechanism. Particles larger than 500 nm are typically phagocytosed or macropinocytosed, whereas smaller hydrophilic molecules are internalized by any of the various endocytotic processes [2, 8]. Often, nanometer-sized transporter molecules or particles accomplish cell uptake simultaneously by several endocytotic mechanisms [9]. Clathrin-dependent endocytosis is the most widely understood pathway, which is present in nearly all mammalian cells. The clathrin pathway is considered the most important uptake mechanism for several polymers such as poly(ethylene glycol)-polylactides, poly(lactide-co-glycolide) (PLGA), silica-based nanoparticles, and chitosan nanoparticles [8, 10–12].

Besides clathrin-mediated cell uptake, caveolae-mediated endocytosis is another well-investigated uptake mechanism. The caveolae pathway is particularly attractive for the delivery of proteins and nucleic acids because these vesicles have neutral pH and the lysosomes, where degradation takes place, are bypassed [8, 13]. Nanoparticles

described to be mainly endocytosed by caveolae include polymeric micelles with a crosslinked anionic core, DOXIL, polysiloxanes, coated quantum dots, and Abraxane [8, 14–17].

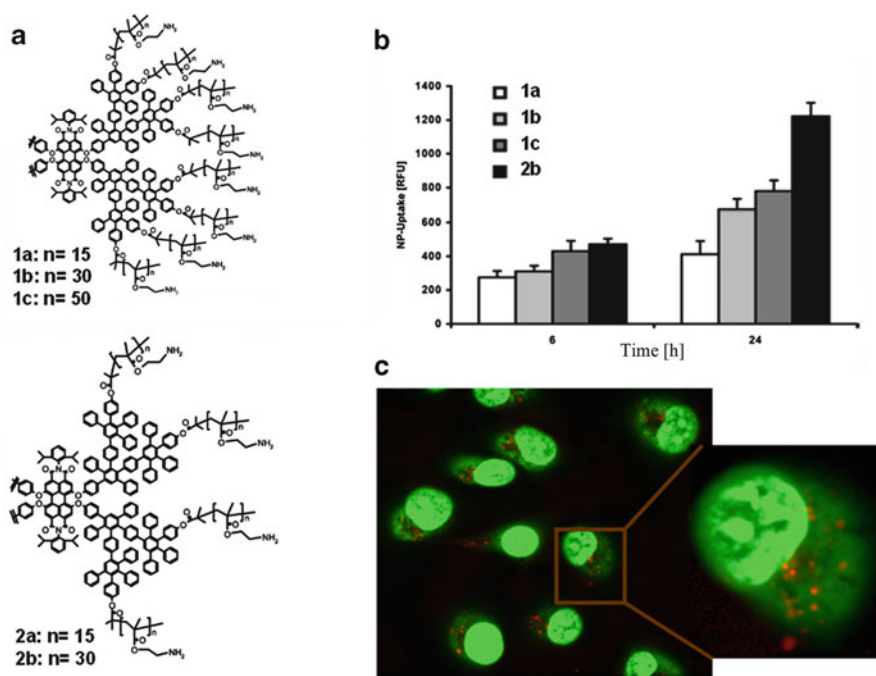
Apart from clathrin- and caveolin-mediated endocytosis, there also exist multiple other pathways independent of clathrin and caveolin-1 that also play an important role in nanoparticle endocytosis. Exploring the clathrin- and caveolin-independent uptake mechanisms has only recently gained attention in endocytosis research, and these less-understood uptake mechanisms might have implications for drug delivery research.

In this chapter, the design of structurally defined dendritic and protein-based polyelectrolyte nanocarriers is presented and the impact of their macromolecular architectures and the presence of multiple charges and charge densities on cellular uptake, trafficking, and cell toxicity is discussed.

## ***1.2 Polycationic and Polyanionic Core–Shell Polyphenylene Dendrimers***

Polycationic proteins such as nuclear histone proteins possess high densities of the positively charged amino acids lysine and arginine located at their periphery, as well as a lipophilic interior. These “natural” polycations are able to complex and to store DNA in the cell nucleus. Based on these structural considerations, dendritic core–shell macromolecules have been designed that possess a lipophilic and relatively stiff polyphenylene scaffold that determines the size and the shape of the macromolecules, as well as a second polymer shell containing multiple positive or negative charges (Fig. 1a) [18]. The position of these charged polymer chains at the dendrimer surface is predetermined by molecular design. The approximate number of charged groups (monomer units) within the polymer chains has been varied by atom transfer radical polymerization (ATRP). In this way, structurally defined macromolecules have been obtained that allow the qualitative correlation of the impact of the architecture and the number and nature of charges of a globular macromolecule on its ability to cross biological membranes [18]. As an additional structural feature, the lipophilic inner part contains a fluorescent perylene-3,4,9,10-tetracarboxdiimide (PDI) chromophore to allow cell uptake studies by fluorescence microscopy (Fig. 1b, c). Polycationic and polyanionic shells have been achieved after polymerization of 2-*tert*-butoxycarbonylaminoethyl methacrylate or *tert*-butyl acrylate, respectively, from the inner polyphenylene core that serves as macroinitiator carrying a defined number of 2-bromo-2-methylpropionic ester groups [18]. After removal of the *tert*-butoxycarbonyl or *tert*-butyl protective groups, the synthesized core–shell macromolecules possesses good water solubility, which is essential for in vitro cell experiments.

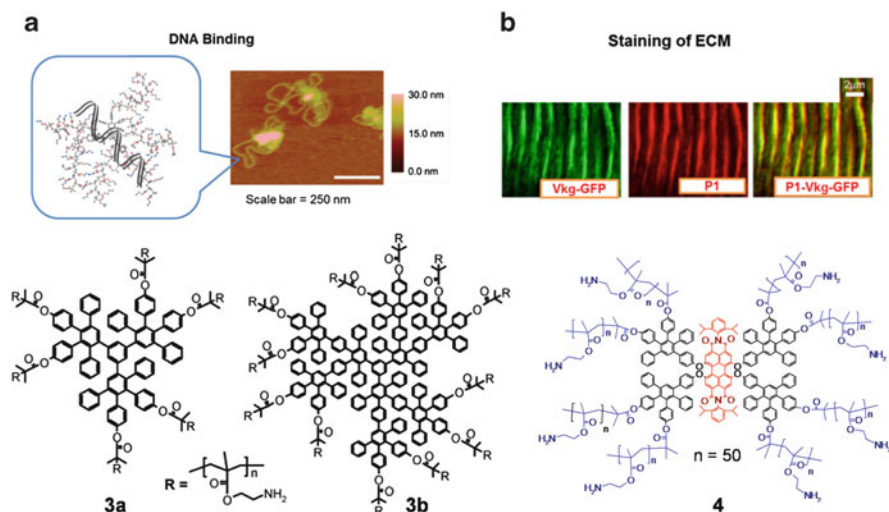
The impact of the macromolecular architecture on cell uptake and toxicity revealed that the presence of positively charged primary amino groups is essential



**Fig. 1** Dendritic core-shell star polymers. (a) Dendritic core-shell star polymers of different architectures with varying densities of the polycationic polymer chains grafted from the dendrimer core as well as varying numbers of positive charges along the individual polymer chains. (b) Cellular uptake of selected macromolecules by ECV-304. The bars represent relative fluorescence units (RFU) measured in individual cells. Data represent mean values ( $\pm$  SEM) after an incubation periods of 6 and 24 h. (c) Cell uptake of polycationic core-shell dendrimers into ECV-304 cells after 15 min. ECV-304 were stained using a green fluorescence cell tracker, whereas the core-shell macromolecules are shown with a red color originating from the PDI core

for efficient membrane uptake, since none of the polyanions was taken up by cells. The total number of grafted polymer chains as well as the polymer chain lengths (e.g., the number of aminoethyl groups per chain) have been varied systematically [18]. Even though all polycations were in principle able to enter cells, increasing the number of cationic repeat units (amino groups) prolonged the time required for cell uptake. In addition, the macromolecular architecture also played an important role. For those polycations with higher densities of polymer chains at the surface of the dendrimers, significantly faster cell uptake was observed but there was also more efficient exocytosis, resulting in lower intracellular concentrations compared to the polycations with lower numbers of polymer chains. In addition, all macromolecules containing a high number and high density of amino groups showed considerable cytotoxicity, most probably due to vesicle leakage and cell lysis [19], which has been reported for other polycationic macromolecules as well.

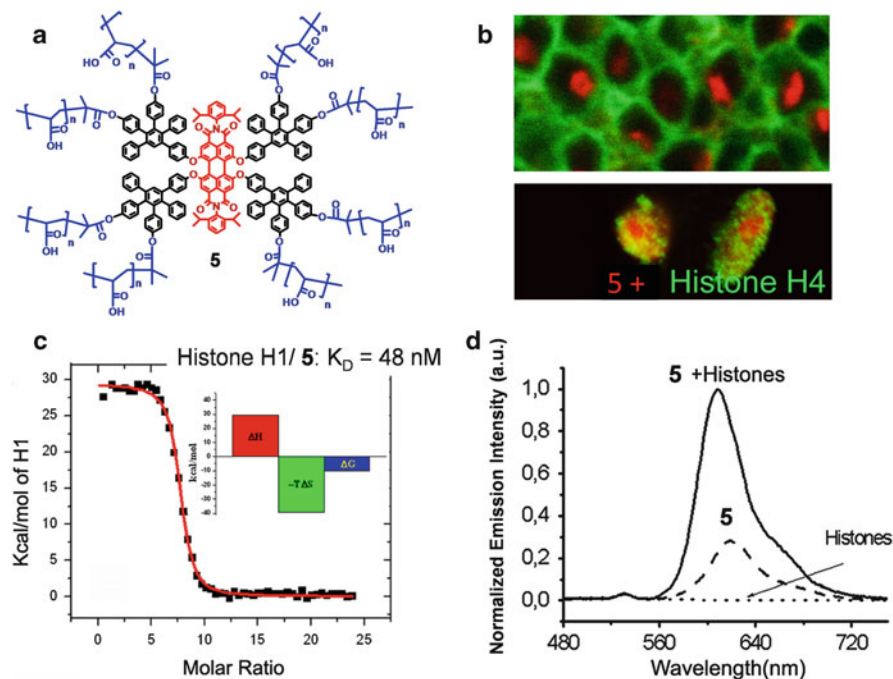
The polycationic core-shell macromolecule **4** (Fig. 2b), formed from a first-generation dendrimer with 50 monomer repeating units, revealed only minor cell



**Fig. 2** Dendritic polyelectrolyte interaction with (a) DNA and (b) the ECM (Vkg-GFP stands for the viking gene that codes for a Collagen type IV protein and localizes to membranes, green emission of the green fluorescent protein, GFP). Structures **3a** and **3b** are first and second generation dendrimers, respectively. Structure **4** is a polycationic red dye (*PI*)

toxicity and was investigated with regard to its potential to interact with subcellular structures [20]. An interaction with the highly negatively charged extracellular matrix (ECM) was observed at physiological pH [20]. Because the ECM surrounds cells and plays an important role in many aspects of cellular fate (including cell migration, stem cell differentiation, and cancer progression) specific labeling of the ECM could be attractive for cell studies. Via affinity analysis, aggrecan was identified as a major interaction partner [20]. Aggrecan is a major structural proteoglycan of cartilage ECM, with a 210–250 kDa core protein to which 100–150 chondroitin sulfate and keratan sulfate chains are attached [21, 22]. In a dot blotting assay, only negatively charged aggrecan was found to interact with the polycationic dendrimer, most probably due to the presence of multiple positive charges. Visualization of the microstructure of the ECM in both fixed and living preparations indicated strong binding to its negatively charged constituents (Fig. 2). Interestingly, the polycationic dye **4** revealed significantly increased emission intensity after binding to the ECM components, and this observation was even compatible with antibody staining, making it useful for multiple channel fluorescence imaging. Due to the observed biological specificity, such polycationic chromophores could serve as an attractive tool for specifically labeling the ECM in life science research [20].

Complex formation of polycationic core-shell macromolecules with natural polyanions such as DNA has been investigated. Three generations of core-shell polymers consisting of a dendritic polyphenylene core and an outer shell containing a defined number of primary amino groups have been synthesized via ATRP (Fig. 2a shows structures **3a** and **3b**, generations 1 and 2, respectively) [23]. All polymers



**Fig. 3** Polyanionic dendrimers interacting with histone proteins. (a) Molecular structure of the first generation polyanionic dendrimer. (b) Confocal scanning microscope images shown the co-localization of polyanionic dendrimer (**5**, red) with histone H4 proteins (green). (c) Isothermal titration graph shows the strong interaction between histone H1 with **5**. (d) Spectral analysis of the **5**/histones

revealed tight binding to different sets of DNA fragments as well as plasmid DNA pUK19 (2,686 bp; 5,372 negative charges). Complex stoichiometries between pUK19 and the polymers were determined by isothermal titration calorimetry and it turned out that the presence of increasing numbers of amino groups within the polymer shell resulted in the formation of polyelectrolyte complexes with higher DNA contents. The average number of dendrimer molecules per pUC19 DNA helix in a complex decreased from 98 (**3a** with about 55 amino groups), to 46 (**3b** with about 116 amino groups) to 30 (third generation dendrimer with about 197 amino groups) under the assumption that neutral complexes were formed [23]. In this way, complex stoichiometries could be controlled by tuning the number of amino groups of the dendritic polycations under the formation of well-defined nanoscopic architectures. Additionally to DNA binding, the question of DNA release was also addressed in this study. A salt concentration-dependent DNA release was achieved with increasing salt concentrations from 50 mM up to 1 M, with complete DNA release at the highest concentrations. These polycationic core-shell dendrimers provide many attractive features such as multiple and adjustable positive charges, allowing high capacity binding and release of nucleic acids, which makes them an interesting tool for DNA staining, gene transfection, and DNA purification.

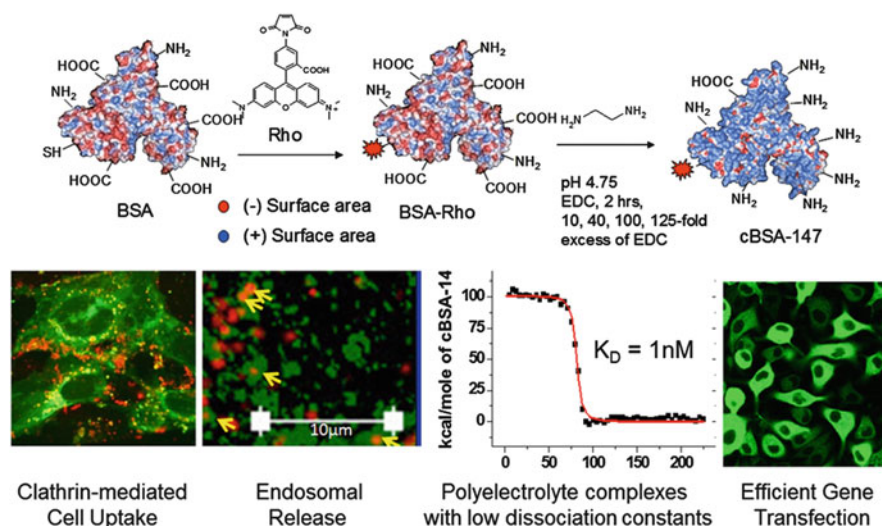


In contrast, polyanionic core–shell dendrimers such as **5** (Fig. 3a) did not display any cellular uptake and, therefore, it was not feasible to investigate the fate of these polyanions inside viable cells [18]. However, applying them to fixed cells allowed cell membrane uptake and specific staining of the cell nuclei in *Drosophila* tissue (Fig. 3b) [20]. In the cell nucleus, DNA wraps around histone proteins forming nucleosomes. The role of histone proteins is to pack and order DNA into structural units based on electrostatic interactions with DNA, which has an important impact for gene transcription. In the presence of the polyanionic core–shell macromolecule **5**, bearing high numbers of negatively charged carboxylate groups, DNA is most probably displaced from the complexes and polyelectrolyte complexes between **5** and the histone H1 proteins are formed due to tight interactions (Fig. 3c). **5** specifically stains the cell nucleus by binding to the positively charged nuclear proteins (Fig. 3d), and it might therefore serve as an attractive alternative to conventional fluorescent antibodies. Polyanionic core–shell dendrimers combine unique properties such as water solubility, high photochemical stability, narrow emission spectra, and cell-specific binding, making them attractive for cytochemical and histochemical studies.

### 1.3 Polycationic Serum Albumin Proteins for Gene Delivery

As discussed above, many synthetic polycations exhibit high cellular toxicities, even at low concentrations. Interestingly, polycationic proteins such as nuclear histone proteins are often much less cytotoxic, which might be due to the presence of many other functional groups within the polypeptide scaffold that potentially “dilute” the effect of the positive charges. In order to assess the impact of the positive charges of polycationic proteins on cell uptake and cell toxicity, polycationic derivatives of the abundant blood plasma protein transporter serum albumin were prepared [6]. Serum albumin is well suited to impart chemical surface modifications due to the high number of negatively charged amino acids (e.g., 100 glutamate and aspartate groups), its diameter of 4.7 nm calculated from the crystal structure [6], and the presence of lipophilic pockets that can accommodate lipophilic guest molecules. Albumin polycations have been achieved by successive conversion of carboxylic acid side chains of the negatively charged amino acids of aspartate and glutamate residues into primary amino groups (Fig. 4) [6]. Stepwise reaction of the carboxylic acid groups with increasing amounts of ethylenediamine and catalyst allowed the synthesis of albumin polycations with varying charge densities. The albumin polycations are denoted according to their modification, e.g., cBSA-147 is cationized bovine serum albumin with 147 additional primary amino groups that have been introduced by chemical modification.

With increasing charge density, more efficient cellular uptake of the polycationic BSA derivatives has been found and the highest polycationic albumin cBSA-147 revealed the most efficient uptake into A549 cells [6]. Interestingly, even at high cBSA-147 concentrations only low cytotoxicity has been found. Cell uptake proceeds



**Fig. 4** *Top*: Cationization of the protein human serum albumin carrying multiple positively charged primary amino groups. Images show that such albumin polycations (stained *red*) reveal efficient cellular uptake by clathrin-mediated endocytosis, endosomal release (*yellow arrows*), and allow gene delivery and release into cells due to tight interaction of DNA, as exemplified by the isothermal titration calorimetry graph

mostly via clathrin-mediated endocytosis, which can be inhibited with chlorpromazine hydrochloride. Rhodamine-labeled cBSA-147 escapes the endosome and is detected in the cytosol as well as in the perinuclear region (Fig. 4). With increasing numbers of primary amino groups, proteolytic stability versus trypsin or proteinase K digestion decreases. Due to the high positive net charge of the albumin polycations, complex formation with plasmid DNA encoding for the green fluorescent protein (pDNA-GFP) has been observed for cBSA-95, cBSA-113, and cBSA-147 derivatives. The latter, in particular, forms stable complexes with plasmid DNA [6]. Isothermal titration calorimetry experiments investigating complex formation were used to assess the relative stoichiometries of the protein/DNA complexes in solution (Fig. 4). Similar to the results observed for the dendritic core-shell polycations, increasing numbers of positive charges facilitated the formation of polyelectrolyte with less cBSA proteins and increased DNA contents. In addition, cBSA-147 binds pDNA-GFP very efficiently and complex dissociation constants in the low nanomolar range (below the detection limit) have been calculated. Varying the ratio of positive (cBSA) versus negative (DNA) charges (P/N) of cBSA and pDNA-GFP has an important impact on complex morphologies: at high P/N rates, DNA is tightly bound and condensed by many polycationic albumins and small, dense complexes as well as low transfection rates have been detected. Equal P/N ratios yield larger complexes containing fewer polycations and high transfection efficacies have been observed (Fig. 4). Most likely, such complexes allow more efficient release of the DNA cargo due to the formation of less densely packed

complexes. This study clearly demonstrates that high numbers of amino groups on cBSA are essential for DNA complex formation but that the number of cBSA molecules in the complexes should be low to facilitate efficient DNA transfection and release. Also, cBSA-147/DNA complexes at low P/N charge ratios reveal considerably reduced cytotoxicity compared to the commercially available transfection reagent Lipofectamine.

In addition, more sophisticated albumin polycation architectures have been designed. Serum albumins offer a single reactive thiol group that is accessible for site-directed chemistry, e.g., Michael reactions with a maleimide functionality that can react exclusively at this position. Two cBSA-147 molecules have been interconnected by a poly(ethylene oxide) (PEO) polymer linker and the successful formation of the cBSA dimers has been visualized by transmission electron microscopy (TEM). These cBSA-dimers revealed efficient cellular uptake as well as transfection of pDNA-GFP.

Furthermore, polycationic albumins have been investigated for the immobilization of viable cells based on electrostatic interactions. cBSA-147, which is readily available on a large scale, allows coating of the walls of a microchannel reactor so that bacterial cells can attach with high cell densities. These whole-cell catalysts are able to enantioselectively reduce ethyl acetoacetate to *R*-ethyl hydroxybutyrate for several days with high productivity and revealed a better profile than the standard polylysine coating [24]. In addition, giant liposomes have been immobilized on cBSA-coated surfaces, which is attractive for in vitro protein synthesis [25]. Coating of primary human cells proved to be less efficient. However, the attachment of cyclic RGD groups to cHSA (cationized human serum albumin) interacting with integrin receptors allowed the immobilization of NIH 3 T3 fibroblast cells, which could have a great impact for, e.g., coating implants with a more biocompatible human protein platform [26].

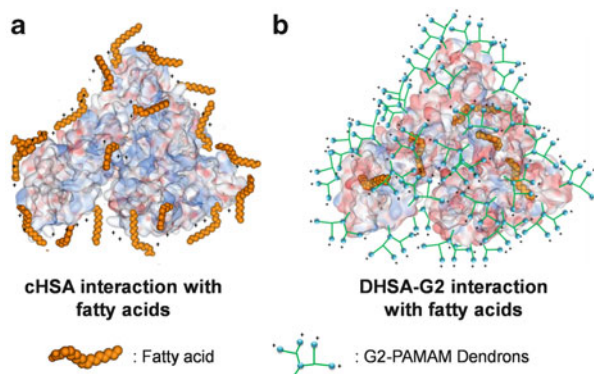
Biopolymers like cBSA offer many reactive groups suitable for multiple surface modifications, making them useful not only for gene delivery but also for drug delivery. Efficient drug delivery is still a high concern of health research because many treatment strategies are still limited by low drug concentrations at the target site or by significant side effects due to high drug doses. Drug delivery systems based on macromolecules such as polymers, dendrimers, or proteins have thus been developed for passive targeting of cancerous cells. These systems make use of the characteristic features of tumor biology that allow the macromolecules to accumulate in the tumor through the enhanced retention and permeation (EPR) effect [27]. The molecular size of the macromolecule plays an important role in tumor cell uptake and the EPR effect is typically observed for macromolecules with molecular weight greater than 20 kDa [28, 29]. Serum albumins are carriers of fatty acids in the blood and, due to their ability to bind to various exogenous and endogenous ligands, they are one of the most exploited proteins for use as a drug delivery vehicle [30, 31] in therapeutics and clinical biochemistry [32], especially for the delivery of lipophilic drug molecules and in particular lipophilic antitumor drugs. HSA drug conjugates are commercially available, e.g., as HSA nanoparticle formulations with the drug paclitaxel (Abraxane) [33]. In addition, HSA–drug conjugates have been achieved that combine the drug molecule and targeting

ligands, and the potential of such modified serum albumins for receptor-mediated delivery of small lipophilic molecules has been investigated thoroughly. In this context, modified BSA and HSA molecules have been described as accommodating small drug molecules such as doxorubicin [34], camptothecin [35], and methotrexate [36].

In order to assess whether polycationic albumins still bear the lipophilic binding pockets that the native proteins offer, electron spin resonance experiments with spin-labeled fatty acids have been carried out [37]. Native HSA and BSA are able to bind about eight fatty acids within their scaffolds and their relative locations have been determined by EPR analysis. After cationization, the flexibility and mobility of the protein scaffold changed significantly, making cHSA considerably less flexible than native HSA. In addition, many fatty acid ligands are bound tightly to the surface of cHSA but none of these molecules has been found in the lipophilic binding pockets (Fig. 5a). It was speculated that the decreased mobility of the albumin scaffold might limit the capacity of cHSA to adapt to the shape of the guest molecules and that a sufficient flexibility might be required for guest uptake. However, it might be also plausible that the fatty acids are trapped on the surface of cHSA due to electrostatic interactions.

In order to dissipate the positive charges within a larger volume, polyamidoamine (PAMAM) dendrons have been attached to the HSA scaffold. PAMAM dendrimers are highly branched, structurally well-defined macromolecules with many primary amino groups within their outer shell. The dendritic branches that form a dendrimer are called dendrons. It has been shown before that PAMAM dendrimers are able to traffic into cells by clathrin-dependent endocytosis [38, 39]. The attachment of about 32 dendrons of the second and third generation to an azido-functionalized albumin by 1,3-dipolar cycloadditions yielded dendronized HSA core-shell macromolecules (DHSA-G2 and DHSA-G3) with positive net charges. Interestingly, EPR experiments indicate the accessibility of the binding pockets of albumin because five or six fatty acids were bound to DHSA-G2 and even to DHSA-G3 (Fig. 5b). In addition, due to the positive net charge of the dendrons, DHSA-G2 revealed membrane uptake, as observed by confocal microscopy. Also, the cytotoxicity of both dendronized core-shell HSA derivatives was considerably lower than that of PAMAM dendrimers with similar numbers of positive charges. This suggests that protein-based polycations and polymer polycations interact differently with cellular membranes, e.g., the latter might have a higher tendency to induce cell leakage due to hole formation in the membrane. The capacity of DHSA-G2 to accommodate and deliver the lipophilic antitumor drug doxorubicin (DOX) has been further addressed [40]. DHSA-G2 reveals a significantly higher loading compared to native HSA since about 11 DOX molecules are bound tightly even in buffer or cell culture medium. In vitro studies indicate a fast cellular uptake of the DHSA-G2-DOX complexes, and DOX release into the cytosol has been substantiated by the observed high cytotoxicity as well as activation of intracellular caspases 3 and 7, which ultimately leads to apoptosis. Therefore, protein dendronization could potentially be considered an attractive strategy for increasing the molecular weight of a protein to

**Fig. 5** Models of cHSA and DHSA-G2. (a) Fatty acids bind on the surface of cHSA. (b) Fatty acids bind to the hydrophobic pockets of DHSA-G2

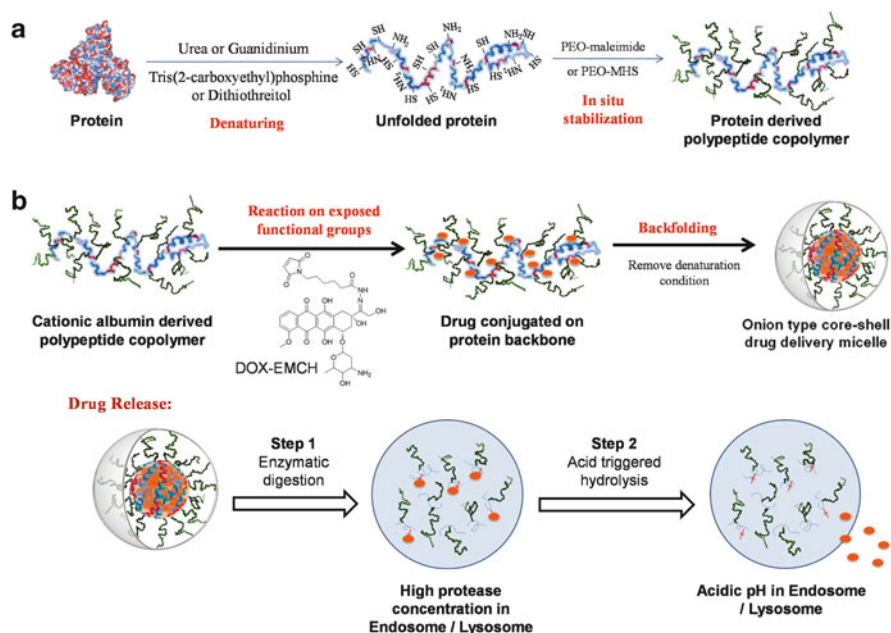


enhance blood circulation, shield epitopes at the protein surface, reduce proteolysis by proteases, and enable drug uptake into the lipophilic interior of albumin.

However, if substantially higher drug loading is required, both the covalent attachment of drugs to the globular HSA protein as well as the non-covalent adsorption into the binding pockets are limited by the available space. In order to further increase the drug loading capacity of HSA, reactive groups within the scaffold of HSA that are normally hidden inside the protein scaffold need to become more accessible for chemical modifications.

#### ***1.4 Albumin Copolymer Polyelectrolytes Allow Efficient Drug Delivery***

Further exploration of multifunctional protein polyelectrolytes requires the full use of the inner functional groups. However, unfolding of a protein's tertiary structure often leads to precipitation and destabilization. Recently, an in situ stabilization method has been developed that allows the unfolding of proteins into polypeptide chains and exposure of "hidden" functional groups. This has been achieved by controlled chemical denaturation of the native proteins and an in situ stabilization with PEO chains (Fig. 6a). Different protein precursors (e.g., HSA, BSA, and the protease lysozyme) have been studied to prove the general applicability of this approach [41, 42]. The protein-derived polypeptide side chain copolymers feature precisely defined backbone lengths, large numbers of readily available functional groups, and partially maintained secondary structure elements [41, 42]. Modification of the exposed functional groups allows successful conjugation onto the protein backbone of multiple reactive moieties such as chromophores, anticancer drugs, or MRI contrast agents [43, 44]. Due to the presence of hydrophobic patches along the protein primary sequence, the unfolded albumin-based polypeptides can efficiently interact with hydrophobic molecules. Polymeric micelles are formed where the hydrophilic groups face to the outside, and hydrophobic drug molecules are encapsulated inside



**Fig. 6** Preparation of core-shell drug delivery micelles. (a) Unfolding of globular protein to prepare protein-derived polypeptide copolymers with exposed internal functional groups. (b) Preparation of onion-type core-shell drug delivery micelles from albumin-derived polypeptide copolymers and the two step release mechanism (adopted and modified from Wu et al. [44])

these micelles [43, 44]. Via this strategy, several polycationic albumin polypeptides have been reported that displayed efficient cell uptake, low cytotoxicity, fast enzymatic degradation, and multifunctionality [41, 44, 45]. Due to their excellent biocompatibility and high drug loading capacity, they have been developed into various drug delivery systems [44, 45].

The hydrophobic effect that leads to micelle formation in the presence of lipophilic guest molecules can be further enhanced by reacting the protein side chains with additional hydrophobic groups such as ethynyl groups [43, 44]. Via this approach, the anticancer drug DOX [44, 45] and hydrophobic fluorophores such as coumarin [43] and perylenediimide [46] have been encapsulated into the interior of these polycationic micelles and efficiently delivered into cells. The DOX-encapsulating albumin micelles showed great potential as macromolecular anticancer drugs due to their fast cellular uptake, significant cytotoxicity, but low toxicity of the albumin carrier [41, 44].

Furthermore, onion-type multishell drug delivery systems offering a two-step controlled drug release have been developed by the conjugation of high numbers of DOX drug molecules into the albumin interior [44] (Fig. 6b). Specifically, HSA has been equipped with a PEO shell to ensure water solubility under denaturing conditions. Then, the HSA backbone is unfolded as described above to expose



cysteine groups for drug conjugation. The anticancer drug DOX is attached to the cysteine residues via an acid-cleavable hydrazone linker. Up to 28 DOX molecules have been uploaded per protein in a reproducible fashion. The protein backbone backfolds after drug loading to form DOX-HSA-PEO multishell micelles with (1) DOX molecules encapsulated inside the core, (2) albumin protein as the enzyme-degradable protecting shell, and (3) PE as water-soluble and immune-silencing shell. This drug delivery system exhibits a controlled two-step drug release mechanism in response to proteolysis and acidic pH environment, therefore reducing undesirable drug leakage. The cell toxicity has been examined *in vitro* as well as *in vivo*. A highly potent toxicity effect in an acute myeloid leukemia (AML) model has been observed, with an IC<sub>50</sub> in the subnanomolar range, and long-term suppression of AML growth has been observed in a mice model [44]. Furthermore, by modifying the multiple functional groups of the protein shell, additional functionalities, such as MRI contrast reagents, could be further attached to this multishell structure. For instance, the most widely used T1 MRI contrast reagent, Gd-DOTA, has been conjugated to the lysine residues of the protein backbone, thus resulting in a theranostic complex that potentially allows therapeutic drug delivery and diagnostic imaging simultaneously (our unpublished data). In the future, this system offers great potential for developing personalized anticancer therapy.

## 1.5 Conclusions

Polyelectrolyte interactions play an important role in cell biology. By introducing multiple positive charges into the periphery of fluorescently labeled polyphenylene dendrimers, charge-dependent membrane uptake and cell toxicity was found. In addition, polycationic dendrimers were able to tightly complex and release DNA and stain the extracellular matrix by interacting with the natural aggrecan polyanions. Dendritic polyanions, in contrast, were not able to pass the cellular membrane; however, in fixed cells, tight binding to histone proteins in the cell nucleus was observed, where DNA was most probably displaced from the complexes. Both the polyanionic and polycationic dendritic chromophores specifically stained cellular structures such as the ECM and the cell nucleus, based on the presence of multiple charges within a small nanoscopic volume. In addition, protein polyelectrolytes have been prepared by converting surface-exposed residues into primary amino or carboxylic acid groups. Albumin polycations revealed considerably lower cytotoxicities than their synthetic analogs as well as efficient cell uptake and intracellular release. Complex stoichiometries with DNA could be adjusted by the charge densities of the albumin polycations. Tight DNA binding and efficient transfection was achieved with the highest cationized cBSA derivative.

Cationization of albumin proteins diminished the accessibility of the albumin binding pockets. However, the attachment of positively charged dendrons allowed guest uptake into the hydrophobic binding pockets and efficient delivery of the cytotoxic drug DOX has been achieved. Denaturing the albumin backbone of

cationic albumin proteins yields polypeptides that can fold back into micelle structures in the presence of lipophilic guest molecules. These micelles can be taken up by cells due to the presence of multiple positive charges along the polypeptide backbone, which makes them attractive for drug delivery applications. A refined polycationic albumin biopolymer has been prepared that bears many drug molecules attached to the polypeptide backbone. These were protected by the polypeptide as well as by a second PEO shell, allowing a controlled two-step drug release. These polycationic polypeptides offer great potential for drug delivery and simultaneous bioimaging and have already been studied in *in vivo* experiments.

## **2 Gene Transfection Utilizing Cationic Cylindrical Brush Polymers**

### **2.1 Introduction**

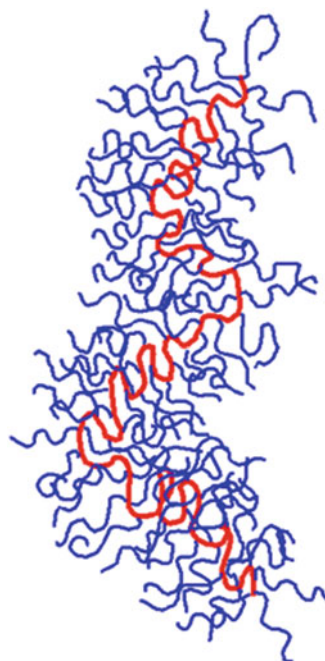
As described above, cationically modified albumin was successfully utilized in gene transfection experiments. A novel cylindrical structure of poly-L-lysine (PLL) in the form of a cylindrical brush polymer with PLL side chains has been utilized for DNA transfection. The results are compared with the transfection efficiency of flexible linear PLL/DNA complexes.

Nonviral gene transfection is a widely used *in vitro* method for studying gene effects in target cells. However, to date only few successful transfections of brain endothelial cells have been published [47–49]. For example, Zhang et al. describe the transfection of murine brain capillary endothelial cells with short polyamines containing a reducible disulfide backbone [48]. Recently, Chen and colleagues described a successful downregulation of expression (60% reduction) via targeted delivery of siRNA-loaded PLGA nanoparticles [50]. Furthermore, another study compared state-of-the-art transfection approaches and determined that only an electroporation protocol achieved efficient transfection rates of brain capillary cells (82% transfected cells) compared to lipid-based transfection (21–40% transfected cells) [49]. Hence, there is still a high demand for the development of nonviral gene delivery systems, especially for difficult-to-transfect cell lines like brain capillary endothelial cells.

Besides viruses [51–57], various synthetic and biological polycation/DNA complexes have also been utilized as vehicles for gene transport [58–66]. Among others, the effects of the polycation chemical composition, charge density, and chain topology (linear flexible coils, hyperbranched or dendritic structures, comb-like polymers) were investigated [67, 68]. Work on PLL with different chain topologies (linear, branched, dendritic) by Mannistö et al. showed no clear relation between the physicochemical properties (complex size, zeta-potential, condensation of DNA) and the transfection efficiency [69]. However, linear PLL was more efficient for transfection of D407 cell lines than the dendritic or branched polymer.



**Fig. 7** Sketch of a cylindrical brush polymer. *Thick red line*, polymethacrylate main chain; *fine blue lines*, PLL side chains. The main chain is forced into an extended conformation due to the strong steric repulsion of the densely grafted side chains



## 2.2 Polymers for Transfection

Rod-like, semiflexible, and ring-shaped polymers were claimed to be promising candidates as drug delivery vehicles, showing prolonged in vivo circulation times [70–73] and excellent accumulation in tumor cells [72, 74]. Reports on complexes of stiff polycations with DNA are rare. Besides other reports [75, 76], pullulan modified with spermine groups was reported to transfect T24 cells of a human bladder cancer cell line [77], HepG2 cells of a human hepatoma cell line [78, 79], and human microvascular endothelial cells [80].

Cylindrical brush polymers [81–88] constitute a prominent class of worm-like cylindrical polymers, which consist of a long main chain (degree of polymerization  $P_n > 500$ ) densely decorated with short polymeric side chains ( $20 < P_n < 100$ ). Due to the extremely large grafting density (typically one side chain per 0.25–0.5 nm main chain contour length) the steric repulsion of the side chains forces the main chain into an extended conformation with a persistence length comparable to that of DNA (see Fig. 7).

Such polymer topologies have not been utilized in DNA transfection studies except for one study by Liu et al. [68], who report on brush-like polymers with PEI-*b*-PEO side chains. However, in these polymers the main chain length was hardly longer than the side chain length, resulting in a spherical rather than cylindrical brush structure.

## 2.3 Brush Polymer Synthesis and DNA Complexation

The PLL-brush polymers were synthesized by grafting Z-protected lysine by ring-opening polymerization from a macroinitiator, as described elsewhere [87]. The total weight-average molar mass ( $M_w$ ) of the deprotected PLL brush was determined in aqueous 5 mM LiBr by light scattering to be  $1.03 \times 10^7$  g/mol, yielding an average of 55 lysine units per side chain [87]. The overall brush dimensions were  $R_g = 84.4$  nm and  $R_h = 56.7$  nm. The degree of polymerization of the macroinitiator was evaluated by light scattering to be  $P_w = 900$ . The polydispersity measured by gel permeation chromatography in dimethyl formamide (DMF) was  $M_w/M_n = 1.5$  (polystyrene calibration). Linear PLL with  $M_w = 326,000$  g/mol,  $R_g = 27$  nm, and  $R_h = 21$  nm was also utilized for complex formation and transfection studies. The DNA/polycation complexes were prepared by adding DNA (pUC19) solution dropwise to a polycation solution.

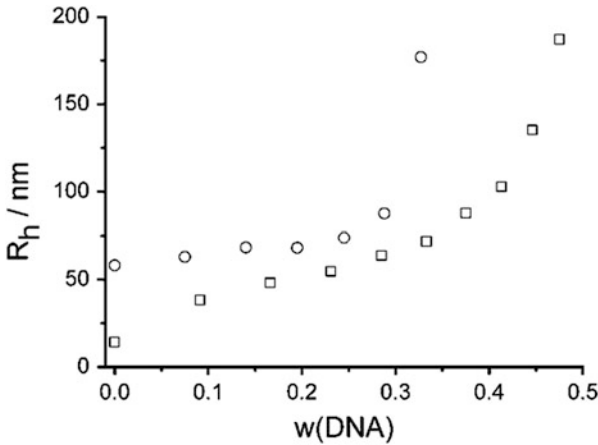
Complexes can be formed by dropwise addition of DNA solution to the polycation solution. At low excess of polycation, the radii of the complex mixture remain small, in the size regime of either the pure polycation or the DNA. In this part of the phase diagram, complexes are known to coexist with excess polycation [89–91]. When approaching the point where the molar mass and the radii of the complexes start to diverge, the excess component becomes fully incorporated into the complexes. Upon further addition of DNA, the solution becomes unstable due to bridging of the primary complexes by additional DNA. These experiments were performed with pUC19 DNA but similar results are to be expected for the larger GFP-DNA (Fig. 8). For linear PLL, this diverging point lies in the equimolar charge regime, whereas it is shifted to a bigger excess of polycation for brush molecules in qualitative agreement with our former results on similar systems [89]. Above  $z^+/z^- = 1.5$  for complexes with linear PLL and  $z^+/z^- = 2.2$  with PLL brushes, no free DNA could be detected.

For transfection experiments, these critical DNA weight fractions ( $w_{\text{DNA}}$ ) limit the choice of mixing ratios to  $0.15 < w_{\text{DNA}} < 0.2$ , because  $w_{\text{DNA}}$  should be well below the diverging point and the solution for transfection should not contain too-large an excess of uncomplexed polycation.

Typically, one complex contains 20 DNA molecules and 10 cylindrical brush molecules, whereas more than 100 linear PLL chains are involved in a complex containing similar numbers of DNA molecules.

For a successful transfection, DNA must be released from complexes. One prominent hypothesis postulates that anionic competitors, most likely polyions, could replace DNA in the complex [92, 93]. Hence, lower molar mass heparin has been established as a model for DNA release experiments, although heparin is not abundant in cells. In addition, the serum protein albumin (which is also not abundant in the cell cytoplasm) as representative of proteins with a small anionic charge, NaCl, and RNA were tested for their ability to release DNA from brush polymers and linear

**Fig. 8** Hydrodynamic radii  $R_h$  of PLL-brush/pUC19 complexes and of linear PLL/pUC19 complexes. The hydrodynamic radii  $R_h$  of PLL-brush/pUC19 complexes (*circles*) and the hydrodynamic radii of linear PLL/pUC19 complexes (*squares*) are shown as function of the weight fraction of DNA,  $w_{DNA}$ . Complexes were prepared in 150 mM NaCl with a total concentration of 10 mg/L



**Table 1** DNA release from polycation complexes and DNase protection

	PLL brush	Linear PLL
Heparin	Release increases with charge ratios starting at $z^-(\text{Heparin})/z^-(\text{DNA}) > 25$	No release for $z^-(\text{Heparin})/z^-(\text{DNA}) < 250$ , larger ratios not tested
BSA	Release for $z^-(\text{BSA})/z^-(\text{DNA}) > 12$	No release for $z^-(\text{BSA})/z^-(\text{DNA}) < 140$ , larger ratios not tested
NaCl	No release (up to 2 M NaCl)	No release (up to 2 M NaCl)
RNA	No release for $z^-(\text{RNA})/z^-(\text{DNA}) < 5.3$ , larger ratios not tested	No release for $z^-(\text{RNA})/z^-(\text{DNA}) < 5.3$ , larger ratios not tested
DNase protection	No	No

Conditions for DNA release from linear and cylindrical brush polycation complexes with different competitors (heparin, bovine serum albumin, RNA) and NaCl. DNase-I-mediated degradation was also measured to see whether complexation gave protection

polymers [94]. Results of the release experiments are summarized in Table 1. Complexes with linear PLL were very stable and DNA was not released by any of the applied competitors. In contrast, complexes with brush polymers were less stable and both heparin and BSA were able to release DNA, depending on the excess of competitor to DNA.

Additionally, it was investigated whether complexation of DNA with polycations could guard DNA from degradation by DNase. Degradation assays with DNase revealed that none of the complexes provided enhanced protection against DNase degradation. Recent reports on DNA protection against DNase

degradation in Lipofectamine/DNA complexes are controversial [95–98] and do not allow a detailed discussion on the importance of DNase protection on the transfection efficiency.

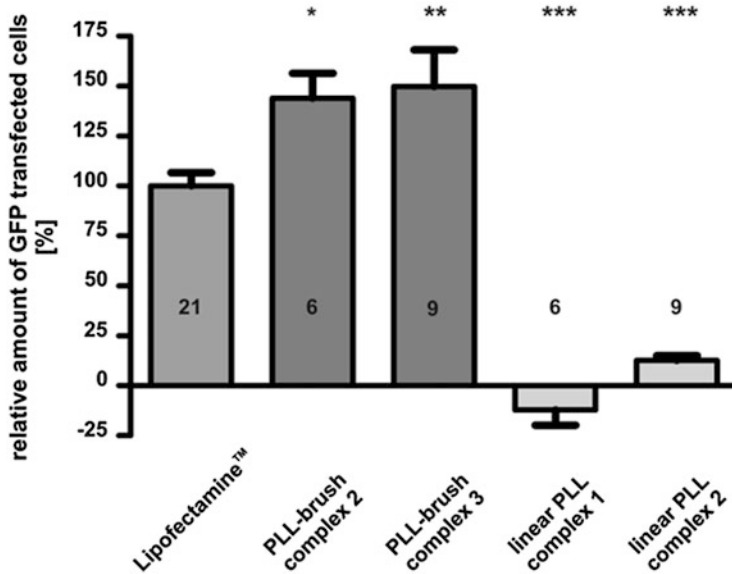
Whereas the release experiments clearly showed that DNA was more easily released from the cylindrical brush complexes than from linear PLL complexes, experiments with ethidiumbromide revealed that all DNA phosphate groups were complexed by the cationic charges for both linear and cylindrical brush PLLs. Thus, subtle topology-dependent enthalpic and entropic effects may play a deciding role in the DNA release out of the complexes.

## 2.4 Transfection with Brush Polymers

Prior to transfection, the question of cell cytotoxicity needs to be addressed. Porcine microvascular endothelial cell (PBMEC) viability was affected in a concentration-dependent manner and was similar for linear PLL and cylindrical PLL brushes, with a 50% cell survival concentration of 0.5 mg/mL. For comparison, the cell cytotoxicity of linear PLLs and stiff cylindrical PLL-brush polymers on fibroblasts (NIH/3T3) was also tested, which is known to be very high for linear PLL [99]. These results could be confirmed because linear as well as brush PLL polymers were found to be very toxic for fibroblasts (50% cell survival concentration 0.01 mg/mL). The differences in cytotoxicity between the two cell lines may be explained by cell type-dependent differences. Brain endothelial cells possess transport systems (efflux transporter of ABC family) characteristic for barrier endothelial cells, which constitute a physical barrier to the brain [100]. The PLL-brush polymers showed a 40% reduction in cell viability at a concentration of 0.1 mg/mL and no effect at a concentration of 0.01 mg/mL. PLL brush concentrations used for transfection were <0.1 mg/mL; hence, only moderate effects on cell viability are to be expected during transfection. Compared to the well-known cytotoxic side effects of other transfection reagents (e.g., lipofectins [101]), the moderate effects on cell viability observed here are judged to be of only minor importance.

For in vitro transfection studies, complexes of DNA and cylindrical brush PLL with PLL side chains were formed at a polycation-to-DNA charge ratio of  $z^+/z^- = 10.6$  and  $z^+/z^- = 6.3$ . [94]. Transfection was controlled by confocal microscopy and quantified by cell cytometry. Only complexes with brush PLL provided significant cell transfection of brain capillary endothelial cells (PBMEC), whereas complexes with linear PLL were not effective, as shown in Fig. 9.

It was demonstrated that cylindrical brush/DNA complexes showed the highest transfection efficiencies, even higher than for Lipofectamine controls. Quantitative analysis revealed that cylindrical brush/DNA complexes formed at an excess of 6.3 and 10.6 positive charges per negative charge ( $z^+/z^-$ ) evoked an increase in



**Fig. 9** Transfection efficiency of PLL-brush/pEGFP-C3. Polymer complexes at various charge-mixing ratios with 1.0  $\mu\text{g}$  pEGFP-C3 on PBME cells: PLL-brush complex 2,  $z^+/z^- = 10.6$ ; PLL-brush complex 3,  $z^+/z^- = 6.3$ ; linear PLL complex 1,  $z^+/z^- = 8.9$ ; linear PLL complex 2,  $z^+/z^- = 6.3$ . Lipofectamine was used as a positive control and GFP expression was measured as % of total cell population, 48 h after transfection

transfection efficiency of  $50 \pm 18.2\%$  and  $44 \pm 12.4\%$  (compared to Lipofectamine) whereas similar complexes with linear PLL did not lead to any significant transfection (Fig. 9), although the size of the complexes was similar ( $80 \text{ nm} < R_h < 150 \text{ nm}$ ).

## 2.5 Conclusions

Developing an effective delivery agent for the transfection of challenging cells like capillary endothelial cells is a major experimental problem in in vitro experiments. The results of the studies described here demonstrate that the worm-like topology of synthetic polycations shows superior transfection results as compared to chemically similar linear polycations. PLL-based polycations are known to be toxic for many cell lines, although PBMEC were shown to survive amazingly high concentrations of PLL, up to 0.1 mg/mL. Accordingly, future work will aim to reduce cytotoxicity, for instance by reducing the number of cationic charges. The cylindrical brush polymers presented here have the additional advantage that they can be prepared from a variety of chemically different biocompatible and biodegradable building blocks. The latter option may eventually lead to in vivo applications, provided that the release of DNA by interaction with serum proteins can be prohibited.

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