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Elucidating the interplay between DNA-condensing and free polycations in gene transfection through a mechanistic study of linear and branched PEI

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ABSTRACT

In the present study we compare LPEI and BPEI characteristics related to DNA condensation and their role as free polycation chains in gene transfection. Using radioactive ^{32}P labeled DNA, we investigated the effect of free PEI chains on the cellular uptake of polyplexes. Our investigations show different properties of BPEI and LPEI polyplexes in condensation and de-condensation processes as well as in cellular uptake, which was tightly correlated with transfection efficiency. In agreement with earlier reports we find all DNA to be condensed at N/P = 3. Further added PEI chains remain free in solution. We found that both the cellular uptake and gene transfection of BPEI polyplexes is much more efficient than LPEI polyplexes at a low N/P ratio of 3 (i.e., without free PEI chains). When N/P is high (10, with 7 portions of free PEI), the LPEI and BPEI polyplexes have similar transfection efficiency even though the cellular uptake of the LPEI polyplexes is significantly lower. In addition, we found that addition of free short or long PEI chains (2.5 and 25 kDa) leads to a comparable gene transfection efficiency.

1. Introduction

The development of safe and effective non-viral vectors in gene therapy would be one of the most important achievements in medicine today and is therefore attracting enormous attention [1,2]. Polyethylenimine (PEI) based vectors are considered the gold standard in polymer based gene delivery [3–6], and many novel vector designs utilize PEIs. One of the important features of this polymer is the high concentration of positively charged nitrogen atoms that facilitates effective binding and condensation of negatively charged DNA [7].

Two different structures of PEI, linear and branched, have been used to construct polyplex systems that have been applied in gene delivery and transfection studies [3,6,8,9]. Linear PEI (LPEI) is composed almost exclusively of secondary amines while branched PEI (BPEI) is composed of primary, secondary and tertiary amines. It is clear that this structural difference affects transfection efficiency and vector toxicity, even though the structure–activity relationship is not well understood [6]. Various polyplexes with a broad range of molecular weights of PEI have been tested in *in vitro* transfection studies and a significant difference in the transfection efficiency is observed [10–12]. Itaka et al. compared the intracellular behavior of LPEI 22 kDa and BPEI 25 kDa based polyplexes and showed that the LPEI/DNA polyplex induced a more effective DNA de-condensation, which is critical for successful gene expression [13]. However, whether LPEI or BPEI are the most effective vectors for providing successful gene expression is controversial as many studies points in opposite directions both *in vitro* and *in vivo* [5,14]. One of the main reasons for this controversy is a lack of understanding of the basic mechanisms of polycation based transfection [6], which limits our ability to improve vector designs.

It is clear that in order to achieve efficient transfection, plasmid DNA (pDNA) needs a carrier system that either allows transport across the plasma membrane or more likely internalization through the endocytic pathways in cells. The latter requires DNA condensation into a tightly packed nanometer sized complex suitable for cellular internalization by endocytosis [14–17]. After endosomal escape, DNA needs to be unloaded either in the cytosol or in the nucleus for further transcription [14,18,19]. Therefore, the balance between being associated extracellularly and dissociated intracellularly is crucial [13]. Previous reports have demonstrated that nearly all DNA is complexed with PEI when N/P ratio equals 3 to 4, where N is the number of polymer nitrogen atoms and P the number of DNA phosphorus atoms [20–22]. However, it is also apparent that transfection efficiency can be enhanced dramatically when the N/P ratio is higher. An important and slightly overlooked

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finding was reported by Wagner and coworkers who found that at a N/P ratio of 6, not all PEI is bound in the polyplex and a free PEI fraction exists [23]. Their work showed that a large part of PEI toxicity originates from this free fraction and that the free fraction greatly enhances transfection efficiency. Wu and coworkers recently showed that polyplexes prepared as N/P = 10, has a condensed PEI fraction that corresponds to N/P = 3 and 7 portions of free PEI [24]. They further showed that this free PEI results in 1000 fold higher transfection efficiency when compared to polyplexes of N/P = 3, where no free PEI is present. This was a clear indication of the interplay between condensed and free PEI in successful transfection [24]. However in this regard, the role of free LPEI or BPEI is not clear in relation to condensation efficiency and a mechanistic investigation could potentially elucidate the discrepancy in earlier reports on the structure–activity relationship of these two classes of polymers. Many earlier studies have attributed the superiority of PEI as a non-viral gene vector to the proton sponge effect due to its high buffering capacity [8,25,26], where it has been hypothesized that the endosome acidification results in PEI induced osmotic pressure leading to swelling and subsequent disruption of the endosomes [3]. However, this theory has not been confirmed with definite experimental evidence and fails to explain a number of findings, e.g. the differences found between PEIs of low and high molecular weight [27].

Instead of focusing on the "proton-sponge" effect, we investigate the effect of differences in cellular uptake between polyplexes composed of BPEI and LPEI of different molecular weight and how this is affected by the presence of free PEI, which is another crucial part in the process of transfection. The total amount of DNA that is internalized is known to be tightly correlated with the final transfection performance [11,28–30]. We utilize radioactivity labeled DNA as a fast, sensitive and highly quantitative method for measuring total DNA uptake in cells and compare the condensation and de-condensation capacity of LPEI and BPEI polyplexes, which relates to both extracellular and intracellular polyplex performance and DNA release kinetics. The cellular uptake of DNA/LPEI and DNA/BPEI polyplexes is examined by use of ^{32}P labeled DNA in the absence and presence of free PEI. By combining this with a luciferase activity assay, we attempt to elucidate the different effects of linear and branched PEI as condensing and free chains, the effect of free chains in enhancing transfection efficiency and the connection to polyplex de-condensation properties.

2. Materials and methods

2.1. Materials and cell lines

Linear PEI with a weight-averaged molar mass of 2500 g/mol and 25,000 g/mol (LPEI 2.5 kDa, LPEI 25 kDa, Polysciences Inc.), branched PEI with a weight-averaged molar mass of 25,000 g/mol (BPEI 25 kDa, Sigma–Aldrich) were used without further purification. Due to earlier reports [31] of potential impurities in the commercial LPEI 25 kDa in the form of incomplete deacylation of *N*-propionyl during synthesis, the polymer was tested by ^{1}H NMR, which revealed that the LPEI 25 kDa used was 97% deacylated. Reporter expression plasmid, pCMV-LUC (sequence available upon request), was made with the endo-free Giga kit from Qiagen GmbH (Hilden, Germany) according to the manufacturer's instructions. PicoGreen and dextran sulfate (DS) were purchased from Invitrogen (USA) and Sigma–Aldrich, respectively. HeLa cells were grown at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, penicillin at 100 units/mL and streptomycin at 100 μg/mL.

2.2. Preparation of PEI/DNA polyplexes

The N/P ratio of the polyplex is defined as the molar ratio of the total number of nitrogen atoms in the PEI segment of the polymer to the number of DNA phosphates. Polyplexes with different N/P ratios were formed by adding a cationic polymer solution to an equal volume of DNA solution. The DNA concentration was kept constant while the PEI concentration was varied with the different N/P ratios. Each resultant solution mixture was first vortexed gently for 5 s and then incubated for 5 min at room temperature before further use.

2.3. PicoGreen assay and dissociation of the polyplexes

The complexation of DNA to linear and branched PEI was investigated by measuring the extent of the quenching of PicoGreen. PicoGreen shows maximum fluorescence when it intercalates with pDNA, thus it can measure if uncomplexed pDNA is present. In brief, polyplexes were prepared as described above at room temperature. The fluorescence of the polyplex dispersions was measured 10 min after addition of PicoGreen. A Victor 3 plate reader (Perkin-Elemer, Skovlunde, Demark) was used to measure the fluorescence (λ_{ex} = 480 nm, λ_{em} = 520 nm). To study the dissociation of the polyplexes by addition of polyanions, increasing amount of dextran sulfate was added to the polyplex dispersions. The resulting mixtures were incubated for 30 min at room temperature. PicoGreen fluorescence assay was used to determine the extent of dissociation of the polyplexes.

2.4. Gel electrophoresis measurement on the complexation and dissociation of polyplex

The complexation of DNA and PEI was further evaluated using gel electrophoresis. Each polyplex solution mixture with a desired N/P ratio was loaded on a 1% (w/v) agarose gel containing ethidium bromide (Sigma) in Tris-Acetate EDTA buffer. The amount of DNA loaded into each well was 0.25 μg in a total volume of 10 μL. The electrophoresis was performed at 100 V for 45 min. DNA bands were visualized under UV. The dissociation of polyplex by dextran sulfate was also examined using gel electrophoresis. Dextran sulfate (40:1, weight ratio of dextran sulfate and DNA) was added to the LPEI 25 kDa and BPEI 25 kDa based polyplex. The mixture was incubated for 30 min before agarose gel electrophoresis.

2.5. Particle size and ζ potential measurements on the polyplexes

Dynamic light scattering and ζ potential of the polyplexes were measured on a ZetaPlus (Brookhaven Instruments Co., Holtsville, New York) at 25 °C with an angle of 90°. The incident beam was a HeNe laser beam (633 nm). The polyplexes was measured in HBG buffer (10 mM Hepes, 5% glucose, PH 7.4). The particle size was determined by three cycles of 1.5 min each. ζ potential of each polyplex sample was carried out by three repeated cycles with 15 runs for each.

2.6. Radiolabeling of plasmids

For cellular uptake determination, 1 μg of pDNA was radiolabeled with 50 μCi [α-^{32}P]-dCTP using a nick translation kit (GE Healthcare, Brøndby, Denmark). Unincorporated nucleotides (less than 40%) were removed with the Qiaquick Nucleotide removal kit (Qiagen). Both procedures were performed according to the manufacturer's protocols.

2.7. In vitro transfection and monitoring of luciferase expression

HeLa cells were seeded in 24 wells plates with a density of 50,000 cells per well 24 h prior to transfection. The DNA/PEI solution mixture with a desired N/P ratio was further diluted in serum-free medium and then added with a final amount of 0.8 μg DNA per well. The medium was exchanged to complete DMEM medium with 10% FBS (0.5 mL per well) 4 h after the gene transfection was initiated by addition of polyplex. In the cases where free PEI was added later than polyplex, the exchange to complete DMEM with 10% FBS, was 4 h after addition of free polymer. After 24 h, cells were harvested and washed once in PBS, lysed in Reporter Lysis Buffer and luciferase activity was measured using the luciferase assay system (Promega, Madison, WI, USA). The standard assay conditions using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) involve injection of 100 μl of assay buffer to a tube containing 20 μl cell lysate, and after 1 s, start recording of luminescence for 10 s. The total lysate protein concentration was measured using a BCA kit (Pierce, IL, USA) with BSA as standard. Luciferase activity is expressed as a relative luminescence unit (RLU) per mg cellular protein (mean ± SD of triplicates).

2.8. In vitro cellular uptake

Cellular uptake studies were performed as described above except that trace amounts of ^{32}P-DNA was added to the DNA solution. The internalization process was terminated by removing the medium and washing the cells twice by PBS containing heparin (to remove surface bound polyplexes). The cells were then lysed in 100 μL lysis buffer (50 mM Na-Hepes, 100 mM NaCl, 1 mM EDTA and 1% Triton X-100) and 50 μL samples were mixed with 5 mL scintillation liquid (UltimaGold, Perkin Elmer, Skovlunde, Denmark). Radioactivity was measured as counts per minute (CPM) using a scintillation counter (Beckman Coulter, Fullerton, CA, USA).

3. Results

3.1. Size and ζ potential measurements on the polyplexes

We investigated polyplexes prepared from LPEI 25 kDa and BPEI 25 kDa in HBG buffer (10 mM Hepes, 5% glucose, pH 7.4) and found that the DNA/LPEI and DNA/BPEI polyplexes have a comparable size and ζ potential, as shown in Fig. 1. At N/P = 1, the polyplexes are larger than those at higher N/P ratios, indicating incomplete condensation of DNA. Thus, more cationic charges are required to finally condense anionic DNA chains because both DNA and PEI are macromolecules with polymer chain rigidity, unlike small electrolytes. The average hydrodynamic diameter of the polyplexes decreases to ~100–150 nm when N/P ≥ 3 for both LPEI and BPEI. Furthermore, for both types of the DNA/PEI polyplexes, the ζ potential changes from negative to positive when N/P reaches between 3 and 6.

3.2. The complexation of DNA/LPEI and DNA/BPEI polyplexes

Previous reports have shown that in the case of PEI polyplexes, complete condensation takes place around N/P = 3–4 [20–22]. In our study, the complexation between LPEI 25 kDa and BPEI 25 kDa with DNA was investigated by using the PicoGreen fluorescence dependency on DNA binding. Fig. 2 shows the decrease in PicoGreen fluorescence with increasing N/P ratio due to PEI condensation of DNA. Thus, the result indicates that DNA molecules are condensed by LPEI and BPEI to such an extent that PicoGreen has less access to the DNA. It's noticeable that BPEI exerts a better condensation capacity in comparison to LPEI, especially at low N/P ratios. The full condensation of DNA at N/P ~3 was also confirmed by gel electrophoresis (Fig. 3), reflected in the retarded mobility of the DNA bands. It should be noted that at N/P = 3, the trace DNA bands are still visible when LPEI 2.5 kDa and LPEI 25 kDa are used to condense DNA, but completely disappear when BPEI 25 kDa is used.

3.3. The de-condensation of DNA/LPEI and DNA/BPEI polyplexes

When the negatively charged dextran sulfate chains are added into the dispersion of the DNA/PEI polyplexes, they also bind to cationic PEI and compete with DNA, leading to the displacement of DNA from the polyplex if added in excess. In a de-condensation experiment, the released DNA was monitored using PicoGreen fluorescence intensity. Fig. 4 shows the relative PicoGreen fluorescence intensity dependence on dextran sulfate concentration in different dispersions of DNA/LPEI and DNA/BPEI polyplexes, where each dispersion contains 2.5 μg pDNA. It is clear that when dextran sulfate is added (50 μg), the fluorescence intensity of the DNA/LPEI polyplexes dispersion is totally recovered, i.e., $I_F/I_{F,0} = 1$, but not the DNA/BPEI polyplexes dispersion, revealing that BPEI chains seems to have a stronger condensation capacity than LPEI. The gel electrophoresis experiment supports such a conclusion, as shown in Fig. 5. Namely, DNA chains are nearly completely released from the DNA/LPEI polyplexes when an excessive amount of dextran sulfate is added (dextran sulfate:DNA (w/w) = 40:1), independent of the N/P ratio, but DNA chains are only partially released from the DNA/BPEI polyplexes when N/P ≥ 3.

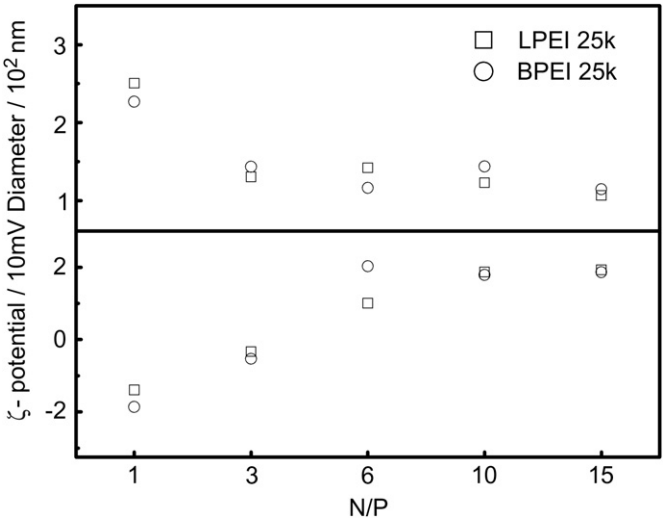

Fig. 1. N/P ratio dependence on size and ζ potential of LPEI 25 kDa and BPEI 25 kDa polyplexes in non-saline buffer.

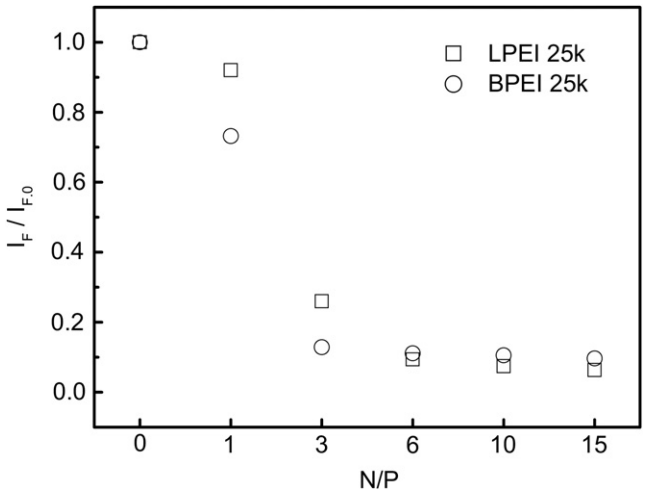

Fig. 2. Complexation profile between PEI (LPEI 25 kDa and BPEI 25 kDa) and DNA at different N/P ratios, evaluated by the PicoGreen fluorescence assay. The amount of DNA is kept constant. I_F and $I_{F,0}$ represent fluorescence intensity of polyplex and free DNA separately. $I_F/I_{F,0}$ displays fluorescence intensity of polyplex normalized by the fluorescence from an equal amount of free DNA.

3.4. In vitro transfection efficiency of DNA/LPEI and DNA/BPEI polyplexes

Wagner and coworkers verified the existence of free PEI when N/P is higher than 6 and discovered the removal of free PEI by size exclusion chromatography brought down the transfection efficiency substantially [23], and showed that addition of free 22 kDa LPEI after addition of polyplex (without the removal of transfection medium) enhanced transfection. Wu and coworkers later elaborated on the importance of free chains in gene transfection and certified the delayed addition of free PEI after polyplex (N/P = 3) administration could improve transfection efficiency substantially [24]. Many research reports have pointed out that the optimum N/P ratio for transfection *in vitro* is between 10 and 15 when using PEIs with molecular weight of 25 kDa [32,33]. Higher N/P ratios may induce the diminution of cell viability as a consequence of PEI cytotoxicity [34,35]. However, previously no adequate conclusions have been offered on the differences in transfection efficiency and toxicity of LPEI and BPEI vectors with similar molecular weight. Some results suggest that at certain N/P ratios, BPEI performs better than LPEI, while others indicate that LPEI is superior [5]. At the same time, the mechanism by which free PEI enhances transfection efficiency is not clear. With the aim of elucidating the differences in transfection efficiency of branched and linear PEI as free chains when administered together with LPEI and BPEI polyplexes, we

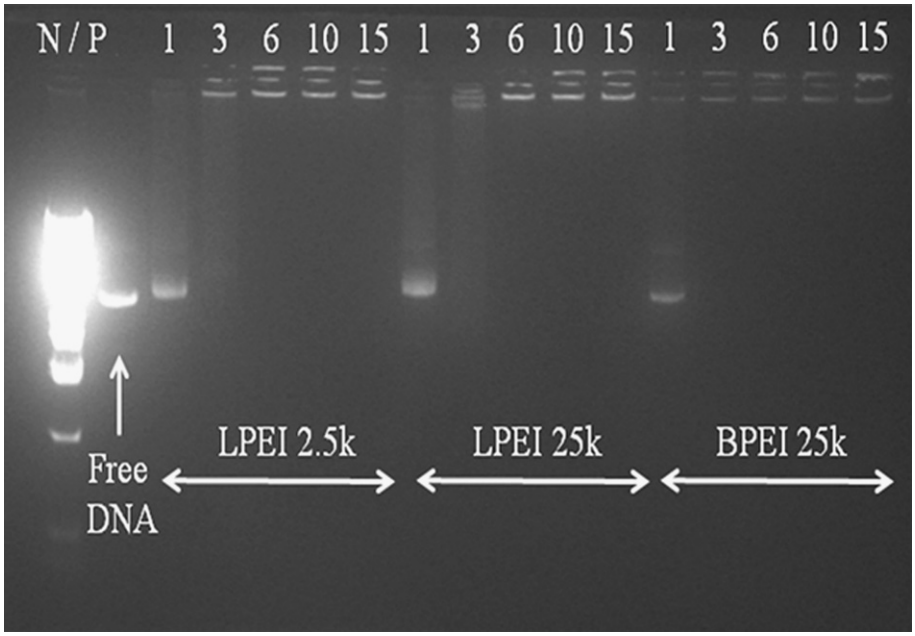

Fig. 3. Complexation profile between PEI (LPEI 2.5 kDa, LPEI 25 kDa and BPEI 25 kDa) and DNA at different N/P ratios, evaluated by gel electrophoresis. The numbers refer to N/P ratios.

investigated three different types of PEI: LPEI 2.5 kDa, LPEI 25 kDa and BPEI 25 kDa.

Fig. 6 shows the luciferase activity of HeLa cells transfected with DNA/LPEI and DNA/BPEI polyplexes. Different types of free PEI are included using a total N/P ratio of 6, 10 and 15. We find that in the absence of free chain PEIs, the branched PEI based polyplex offers better transfection efficiency. This difference is also clear when the amount of free PEI incorporated is low (3 portions, total N/P ratio = 6). Once the availability of free PEI is higher (7 or 12 portions), LPEI and BPEI shows similar transfection efficiency. Thus, this study confirms that the incorporation of free chains enhance the transfection efficiency remarkably [23]. Furthermore, when examining the transfection enhancing effect of different free PEI chains, we observed that free BPEI surpasses LPEI when the total N/P ratio is low, especially when combined with DNA/LPEI polyplex. At higher N/P ratios (N/P = 10 and 15), all the tested free PEIs possess comparable transfection enhancing capacity on both types of polyplexes.

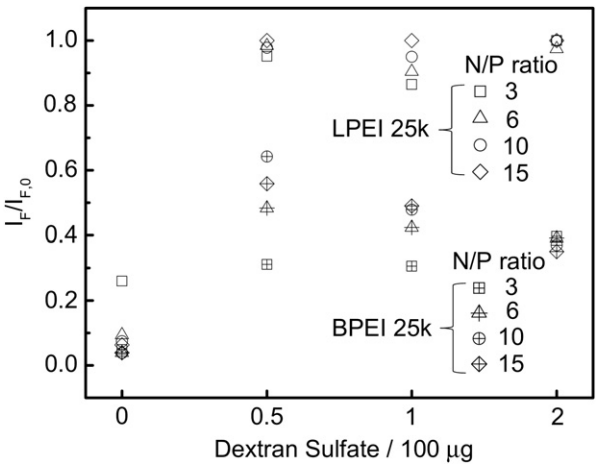

Fig. 4. PicoGreen fluorescence assay of LPEI 25 kDa and BPEI 25 kDa based polyplexes at different N/P ratios. Increasing amounts of dextran sulfate was added to the dispersions (2.5 μg DNA) and incubated for 30 min before measurement. $I_F/I_{F,0}$ displays fluorescence intensity of polyplex is normalized by measuring the fluorescence from an equal amount of free DNA.

We also tested the effects of adding the free PEI chains 2 h or 4 h after addition of the polyplex, i.e. a polyplex of N/P = 3 is added at time zero and additional free PEI is added 2 h or 4 h hereafter (Fig. 6). The efficiency decreases gradually with time between addition of polyplex and free PEI chains, but the transfection efficiency remains high in comparison to the control where no free PEI is added. As for the addition of free PEI chains at 0 h (added together with the polyplexes), the later addition of free chains does not change that free BPEI improve the transfection efficiency more efficiently than free LPEI when the polyplex is based on LPEI, particularly at low N/P ratios. It is furthermore worth noticing that the small and non-toxic 2.5 kDa PEI is relatively effective in promoting transfection when added as free PEI when the polyplexes are formed by longer PEI chains.

3.5. In vitro cellular uptake of DNA/LPEI and DNA/BPEI polyplexes

It is well accepted that the degree of polyplex internalization in cells, e.g. by endocytosis, is tightly connected to the final transfection efficiency. We have used radioactive ^{32}P labeled DNA to investigate cellular uptake, which provides a highly quantitative method to determine the degree of internalization. Fig. 7 demonstrates the cellular uptake of DNA/LPEI and DNA/BPEI polyplexes (N/P = 3) alone and with the addition of 7 portions of free PEI. Polyplexes are added at time zero and cells are harvested and the radioactivity measured at different time points. In all cases, the uptake of polyplex increase with the incubation time as expected. Interestingly, the cellular uptake is enhanced dramatically with the addition of free PEI chains. Fig. 8 compares the effect of free PEI chains (LPEI 2.5 kDa, LPEI 25 kDa and BPEI 25 kDa) on DNA/LPEI and DNA/BPEI polyplexes. Without addition of free chains, DNA/BPEI polyplex results in higher cellular uptake. The inclusion of free PEI chains diminishes the gap in cell internalization between DNA/LPEI and DNA/BPEI polyplexes. However, it remains clear that BPEI generally induces a higher cellular uptake.

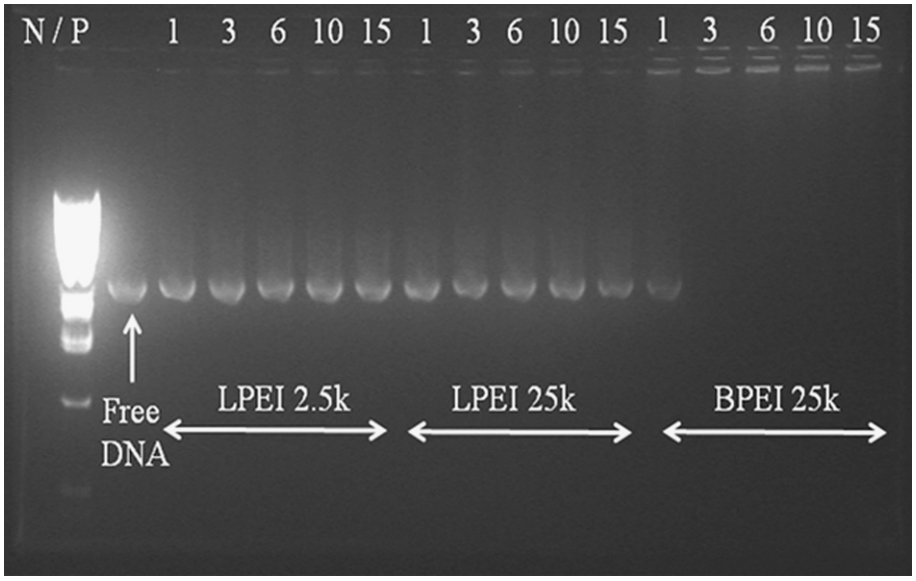

Fig. 5. De-condensation of LPEI 25 kDa and BPEI 25 kDa polyplexes at different N/P ratios with dextran sulfate (40:1, weight ratio of dextran sulfate to pDNA), evaluated by the gel electrophoresis assay. LPEI 2.5 kDa based polyplexes are also examined. The numbers refer to N/P ratios.

We tested 3, 7 and 12 portions of free PEI, using the three different PEI types, which were added at the same time (0 h) or 1 h, 2 h, 3 h or 4 h after addition of the polyplexes. The total cellular uptake of polyplex bound DNA was evaluated 6 h after the addition by use of the radioactivity assay (Figs. 9 and 10). For both DNA/LPEI and DNA/BPEI polyplexes, the delay in addition of free PEI leads to a decrease in cellular uptake when compared to the co-administration, which thereby follow the same trend as the transfection efficiency. In the case of DNA/LPEI polyplex, the uptake enhancing effect of free PEI is higher for BPEI than LPEI when using a low total N/P ratio (N/P = 6). When increasing the amount of free PEI, the disparity diminishes gradually. In the case of DNA/BPEI polyplex, the promotion effect of free branched chains on cell internalization is more pronounced than for the other two types at lower N/P ratios (N/P = 6). The three types of PEI show similar promotion of cell uptake, when the total N/P ratio is 10 or 15.

3.6. Effect of free PEI chains on gene transfection after removal of polyplexes

To further clarify the influence of free chains on polymer transfection, 7 portions and 12 portions of free PEI were added 4 h after addition of polyplex, where the non-internalized polyplex was removed before addition of free PEI by washing (the cells were washed with a heparin solution to remove surface bound polyplex). Cells were hereafter incubated for another 2 h and after which the medium was exchanged with complete DMEM medium with 10% FBS and the cells were incubated for 24 h. Fig. 11 reveals that the luciferase activity decreases dramatically for DNA/LPEI polyplex, probably as a consequence of poor cellular uptake of LPEI based polyplex when free PEI is not present. In contrast, DNA/BPEI polyplex is affected to a much lower degree as the BPEI is capable of inducing relative high cellular uptake even at a N/P = 3 ratio.

4. Discussion

Both DNA condensation and de-condensation contribute to the efficiency of polyplex transfection. While DNA has to remain associated with its carrier as long as it is outside the cells, intracellularly it has to be released for further transcription. Thus, there is a delicate balanced interaction between DNA and PEI, which is critical for successful gene transfection. While the DNA/LPEI and DNA/BPEI polyplexes have a similar hydrodynamic size and ζ potential at different N/P ratios, the fluorescence quenching and

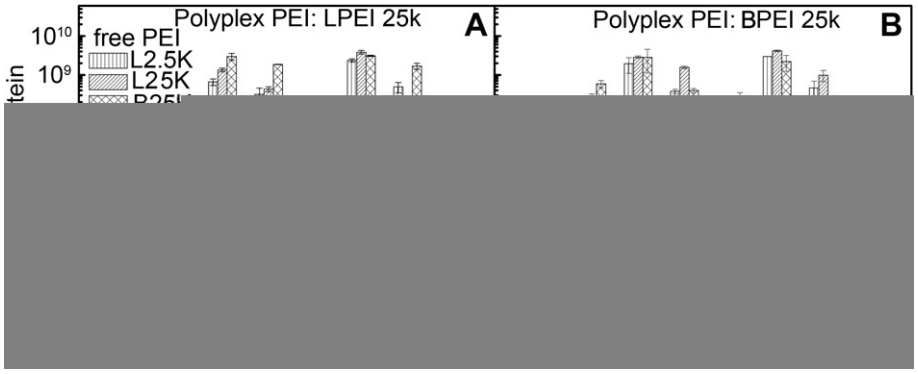

Fig. 6. In vitro transfection efficiency of LPEI 25 kDa and BPEI 25 kDa polyplexes (N/P = 3) in the absence and presence of free PEI chains. Three types of free PEI (LPEI 2.5 kDa, LPEI 25 kDa and BPEI 25 kDa) are administrated simultaneously (0 h), 2 h or 4 h post-polyplex addition to make the total N/P ratio 6, 10 and 15. The different filling patterns represent the different types of free PEIs. The numbers below show the total N/P ratios and time point of addition. (A) Transfection efficiency of LPEI 25 kDa polyplex. (B) Transfection efficiency of BPEI 25 kDa polyplex. Results are presented as mean values ± SD.

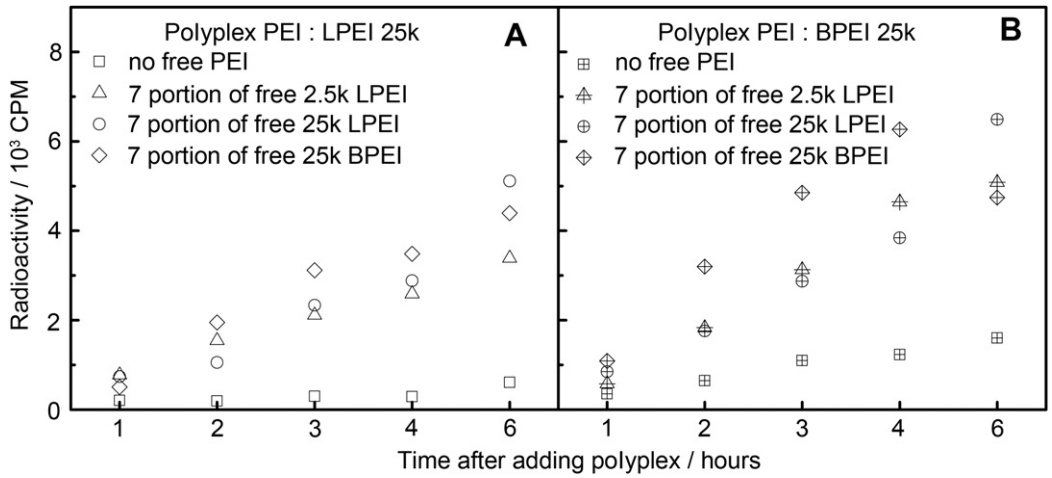

Fig. 7. Effect of free PEI chains on the cell internalization of LPEI 25 kDa and BPEI 25 kDa polyplexes evaluated by scintillation counting of ^{32}P labeled DNA. HeLa cells were incubated with LPEI 25 kDa and BPEI 25 kDa based polyplexes alone (N/P = 3) or in combination with 7 portions of free PEIs (three types), and then harvested and analyzed at the indicated time points. (A) Cellular uptake of LPEI 25 kDa polyplex. (B) Cellular uptake of BPEI 25 kDa polyplex.

de-quenching assay provides important information regarding the difference in their integration and disintegration properties. It is not clear why the DNA/LPEI polyplexes have a lower cellular uptake than the DNA/BPEI polyplexes; however, this effect may be related to a more unconsolidated structure of the DNA/LPEI polyplexes. On the other hand, the less protected DNA chains in the polyplexes made of LPEI are more readily released after cellular internalization. Therefore, there is a tradeoff between the cellular uptake in the extracellular space and the DNA release in the intracellular space.

We have studied the luciferase activity in cultured cells transfected with DNA/LPEI and DNA/BPEI (25 kDa) polyplexes and the effect of free PEI chains (Fig. 6). When polyplexes at low N/P ratios are applied (N/P = 3), without addition of free PEI, DNA/BPEI polyplex shows a remarkable higher transfection efficiency than DNA/LPEI polyplex. However, when 3, 7 or 12 portions of free PEI are added simultaneously (0 h), or 2 h or 4 h post addition of the polyplexes, there is a dramatic effect in transfection efficiency. First of all, we find for both DNA/LPEI and DNA/BPEI polyplexes, that the luciferase expression is boosted significantly in the presence of free PEI. When the total N/P ratio is 10 or 15 (7 or 12 portions of free PEI added), the efficiency of DNA/LPEI polyplex is equal to the BPEI based one. Secondly, we noted that the delay of adding free PEI reduced the efficiency gradually but it remained significantly better than conditioning without free chains. Thirdly, we found that BPEI 25 kDa is much more effective than the other two linear ones when the total N/P ratio is low or when free PEI is added later than the

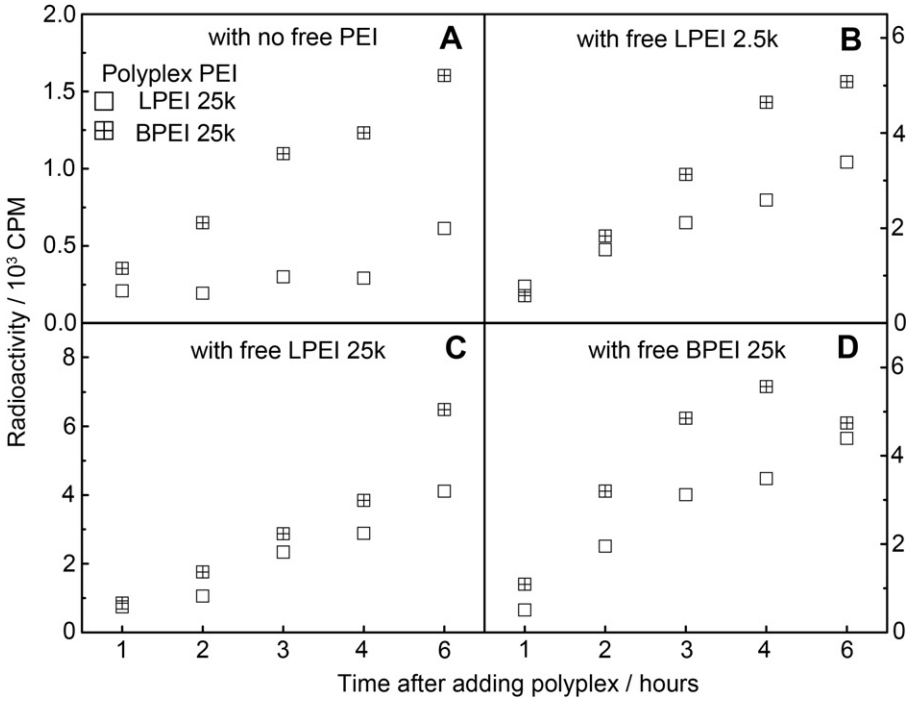

Fig. 8. Comparison of cellular uptake of LPEI 25 kDa and BPEI 25 kDa polyplexes alone (N/P = 3) and in the presence of 7 portions of free PEIs at different time points after addition of polyplex. (A) Cellular uptake of polyplexes without free chains. (B) Cellular uptake of polyplexes with 7 portions of LPEI 2.5 kDa. (C) Cellular uptake of polyplexes with 7 portions of LPEI 25 kDa. (D) Cellular uptake of polyplexes with 7 portions of BPEI 25 kDa.

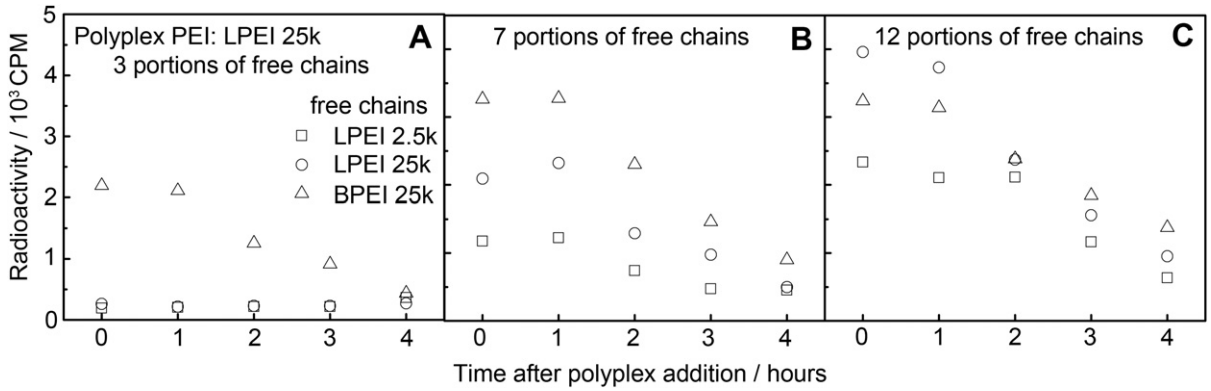

Fig. 9. Cellular uptake of LPEI/DNA polyplex with various amounts of free chains (three types) added at different time points. The Polyplexes are added to cells at time zero. 3, 7 or 12 portions of free chains (three types) are added simultaneously (0 h), 1 h, 2 h, 3 h or 4 h post-polyplex addition. Cells are harvested at 6 h post-polyplex addition and uptake is evaluated by scintillation counting of ^{32}P labeled DNA. (A) Cellular uptake of LPEI/DNA polyplex with 3 portions of free chains. (B) Cellular uptake of LPEI/DNA polyplex with 7 portions of free chains. (C) Cellular uptake of LPEI/DNA polyplex with 12 portions of free chains.

polyplexes. In an attempt to understand these phenomenons, our focus was directed toward the first step of transfection, cellular uptake.

As the first step in transfection, cellular uptake of polyplexes determines the amount of DNA loaded into cells. Although the polyplex has to survive and escape the harsh conditions in the endosome–lysosome pathway and disassembly needs to occur for successful transcription, it is obvious that cell internalization is a vital part of transfection. From the ^{32}P scintillation assay, we found that the total degree of cellular uptake correlates well with transfection efficiency measured by luciferase activity. For all samples, the cellular uptake goes up as the incubation time increases. Regarding polyplexes alone (N/P = 3), the DNA/BPEI polyplex display a much higher internalization in comparison to LPEI. Cellular uptake was enhanced conspicuously with the addition of free PEI for both cases and thereby correlates with the transfection efficiency. When 7 portions of free PEI are added simultaneously as the polyplexes (total N/P = 10), the differences in cellular internalization between DNA/LPEI and DNA/BPEI polyplexes is diminished remarkably for all the used free PEI types (Fig. 8).

Cell internalization was also analyzed with free PEI addition at different time points after addition of polyplex to the cells (Fig. 9). In all the tested combinations, the post addition of free PEI gave a lower total cellular uptake of polyplex when compared to simultaneous addition. In relation to both DNA/LPEI and DNA/BPEI based polyplexes, it is noticeable that with increasing amounts of free PEI, the amount of polyplexes that are internalized by cells rise concomitantly for all the free PEIs investigated. Also, we notice that internalization is improved more by branched chains than linear chains when the total N/P ratio is 6 and 10. This advantage vanishes when the amount of free chains is relatively high (total N/P = 15), which is probably influenced by a degree of BPEI cytotoxicity at this concentration of free BPEI due to high cellular uptake. When it comes to the DNA/BPEI polyplex, the branched chains are superior in inducing cellular uptake when N/P ratio is low (N/P = 6). It is surprising to see that when the amount of free PEI is sufficient (N/P = 15); LPEI 2.5 kDa bears an enhancing effect on cellular uptake that is comparable to the other two longer 25 kDa PEIs as free chains, which correlates to the transfection efficiency. Earlier reports have pointed out that the optimum molecular weight for PEI as vectors is around 20–25 kDa [12,36,37]. The poorer performance of low molecular weight PEI has been ascribed to unstable constructs with DNA and a low endosomal release capacity [8,38]. Our results indicate that low MW PEI may provide a comparable release from endosomes as high MW PEI and it may be the cellular uptake that causes the differences observed in low and high MW PEI transfection efficiency, due to differences in DNA compaction and polyplex stability. This result provides some support to the proton sponge hypothesis. Even though this effect is heavily debated, one of the weaknesses of the hypothesis has been that the osmotic pressure generated by smaller PEI in principle should be

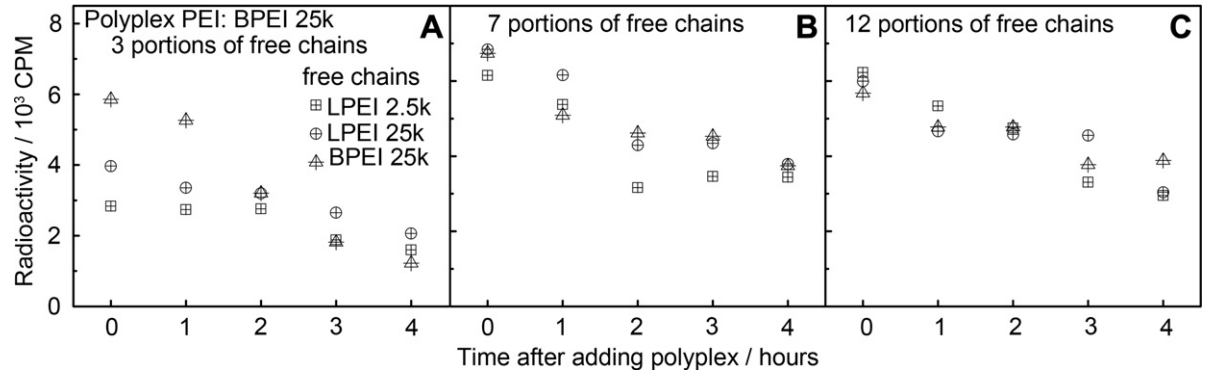

Fig. 10. Cellular uptake of BPEI/DNA polyplexes with various amount of free chains (three types) incorporated at different time points, evaluated by scintillation counting of ^{32}P labeled DNA. Cells are transfected with polyplex at time zero. 3, 7 or 12 portions of free chains (three types) are added simultaneously (0 h), 1 h, 2 h, 3 h or 4 h post-polyplex addition. Cells are harvested and analyzed 6 h post-polyplex addition. (A) Cellular uptake of BPEI/DNA polyplex with 3 portions of free chains. (B) Cellular uptake of BPEI/DNA polyplex with 7 portions of free chains. (C) Cellular uptake of BPEI/DNA polyplex with 12 portions of free chains.

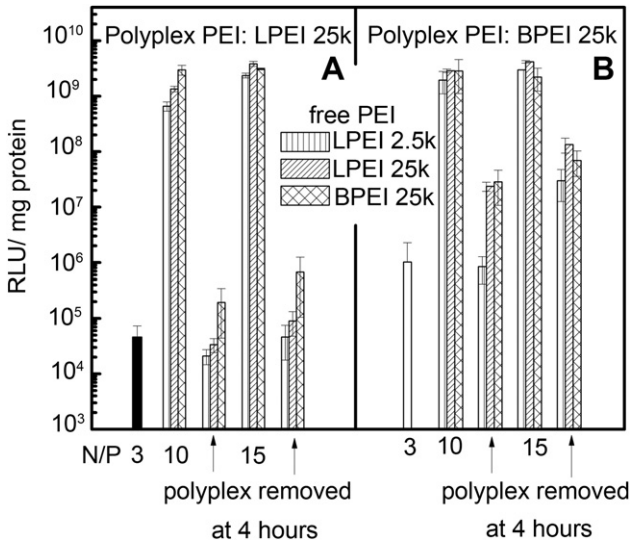

Fig. 11. Effect of free PEI chains on the transfection efficiency of LPEI and BPEI polyplexes, where 7 portion and 12 portions of free PEI (three types) chains were added 4 h post-administration of polyplexes (N/P = 3) after exchanging the cell culture medium and washing the cells with heparin to remove cell surface bound polyplexes. (A) Transfection efficiency of LPEI 25 kDa based polyplex. (B) Transfection efficiency of BPEI 25 kDa based polyplex. Results are presented as mean values ± SD.

higher than for a large PEI (the buffering capacity is the same at a given N/P ratio). However, as literature studies agree that e.g. 25 kDa PEI provides better tranfection efficiency than 2.5 kDa PEI, there is an argument that the proton sponge effect cannot be the dominating factor in endosomal release. Our data suggest that it is mainly the polyplex internalization that is lower for the low molecular weight polycations and the proton sponge effect could therefore still be a reasonable hypothesis. It is well known that the cytotoxicity of PEI grows with increasing molecular weight [39], and from this perspective it is highly interesting that it is possible to add low MW PEI as free chains to polyplexes constituted of high MW PEI and obtain a transfection efficiency that is comparable to using high MW PEI exclusively. It is clear that this will provide an advantage in terms of lowered toxicity and this should be investigated in detail in future studies, even though the toxicity of different MW PEIs is already well established [6].

The different behavior of DNA/LPEI 25 kDa and DNA/BPEI 25 kDa polyplexes in cellular uptake is correlated with their different performance in transfection efficiency. The more compact structure of DNA/BPEI polyplex, as evident by the employed dextran sulfate assay, apparently makes it exceed DNA/LPEI polyplex performance in cellular internalization. In the experiments where free PEI is added after removal of the polyplexes (Fig. 11), the luciferase activity diminished for both DNA/LPEI and DNA/BPEI based polyplexes. However, the BPEI based one is affected to a much lower degree than the LPEI based polyplex, due to the better cellular internalization of the DNA/BPEI polyplex alone (at N/P = 3). The result shows that the free chains dramatically influence transfection efficiency of already internalized polyplexes, and we hypothesize that the free chains influences endosomal escape of already internalized polyplexes. It is noteworthy that at low N/P ratio (total N/P = 6) free chain BPEI promotes uptake and transfection to a significantly higher extent than the LPEI, indicating that free PEI may interact with the polyplex.

As discussed earlier, it is clear that free PEI in general induce higher cellular uptake. One interpretation of this is that the free PEI weakly associates with the polyplexes and enhances the surface charge of the complex giving higher affinity for the negatively charged cell membrane. At N/P = 3 the polyplexes are close to neutral and the cationic polyplexes can associate with the polyplexes and increase their charge. However, the zeta potential measurements do not fully support this hypothesis as the cell uptake increases considerably when going from 3 to 7 portions (total N/P = 6 and 10) of free PEI (Fig. 9) and the zeta potential of the polyplex does not seem to increase in this range (Fig. 1). Also, we have tried adding 10% serum to the solution in the cell uptake studies (data not shown) to see if this would inhibit the effect due to free polymer association with serum proteins, but the effect remained the same. We therefore speculate that the increased cellular uptake also originates from cell membrane perturbing effects that may lead to enhanced internalization of polyplex by the cell (e.g. by macropinocytosis) or direct polyplex translocation due to loss of cell membrane integrity.

It is interesting that even with high concentrations of free PEI, the internalization of DNA/BPEI polyplex is significantly higher than for the DNA/LPEI polyplexes, which is evident from the ^{32}P assay (compare Figs. 9 and 10). However, the difference in cellular uptake at high N/P ratios does not result in a difference in transfection efficiency. We hypothesize that the internalization is a major factor in transfection, but once a high uptake is achieved the endosomal escape and ease of de-condensation becomes important, where LPEI could be superior to BPEI. Itaka et al. [13] studied intracellular trafficking of polyplexes and plasmid DNA de-condensation including linear and branched PEI using confocal microscopy and fluorescence resonance energy transfer (FRET). They observed that DNA/LPEI polyplex underwent a rapid escape from the endosome, spreading uniformly into the cytoplasm with a substantial decrease in FRET efficiency due to the disintegration of DNA/LPEI polyplex structure, whereas DNA/BPEI polyplex retains an appreciable stability even after 24 h. The PicoGreen fluorescence and gel electrophoresis experiments with dextran sulfate conducted in the present study (Figs. 4 and 5) supports their findings indirectly. Thus, we hypothesize that after cellular uptake, an effective disintegration of DNA/LPEI the cytoplasm is likely to be the determining factor for the excellent transfection efficiency of LPEI at high N/P ratio, and it is the higher overall internalization of BPEI complexes that makes this polymer equally efficient at high N/P ratios. Thus, at lower N/P ratios where BPEI is superior, the DNA/LPEI polyplex is simply not internalized to a sufficient degree. The higher overall uptake of DNA/BPEI polyplex also explains the higher observed toxicity for this polymer [6,17].

5. Conclusion

We have shown that DNA/BPEI polyplexes have a higher cellular uptake rate than the DNA/LPEI polyplexes at low N/P ratios. When adding free PEI chains together with polyplexes, the cellular uptake is significantly increased, which illustrates one of the roles that free PEI chains plays in gene transfection. Free 25 kDa BPEI was especially effective in enhancing polyplex internalization. The reason for the uptake promoting effect of free PEI is not clear but could involve interactions with polyplexes resulting in higher surface charge or cell membrane perturbation effects. Interestingly, we found that addition of free LPEI 2.5 kDa has a comparable promoting effect as LPEI 25 kDa and BPEI 25 kDa, with respect to transfection efficiency, when the total N/P ratio is 10 or 15. More efficient non-viral gene transfection systems could therefore be constructed by using high MW PEI for polyplex formation in combination with free chain PEIs with low MW, as low MW PEIs are associated with a lower cytotoxicity. The results provided impacts our design of polycationic transfection systems, as the role that free PEI is not easily transferred to *in vivo* transfection systems and we should therefore reconsider some *in vivo* strategies. Furthermore, it is conceivable

that the reason why BPEI has been reported to be more toxic than LPEI is due its higher cellular uptake and not as a consequence of the difference in structure of the two polymers in relation to their membrane perturbing effects. Future studies should elucidate the mechanism of toxicity in more detail.

Acknowledgments

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