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Difference in the core-shell dynamics of polyethyleneimine and poly(L-lysine) DNA polyplexes.

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ABSTRACT

Electrostatic polymer-DNA complexes (polyplexes) have been widely investigated for DNA delivery, and remarkable differences in transfection efficacy have been seen among the materials. For example, polyethyleneimine (PEI) mediates DNA transfection more effectively than poly(L-lysine) (PLL). Biophysical properties of the polyplexes may explain their different properties in gene delivery. We investigated the structural dynamics in DNA polyplexes, especially the material exchange between the core and shell regions of the PEI and PLL polyplexes. Steady-state fluorescence spectroscopy and double labeling based fluorescence resonance energy transfer (FRET) techniques were used to study the DNA polyplexes. According to our results there is a clear difference between these two polymers: core exchange takes place in PEI but not in PLL polyplexes. Such differences in structural dynamics of polyplexes explain, at least partly, the differences in DNA release and transfection efficacy at cellular level.

Key words: Polyplex; DNA complexation; fluorescence spectroscopy; non-viral gene delivery; polyethylene imine; poly-L-lysine
1. Introduction

Polyethyleneimine (PEI) forms complexes with DNA (polyplexes) spontaneously and delivers DNA effectively into the cells thereby providing efficient DNA transfection (Boussif et al., 1995). Interestingly, the effective transfection with PEI polyplexes requires high ratios of amino groups of PEI to the phosphates of DNA (N/P ratios), whereas at lower N/P ratios PEI shows low efficacy of DNA delivery (Ruponen et al., 1999). Compared to many other polycations, such as poly-L-lysine (PLL), PEI shows high levels of DNA transfection (Ruponen et al., 1999), and the higher efficacy has been attributed to the buffering capacity of PEI within the endosomal compartment (Boussif et al., 1995). Indeed, PEI has primary, secondary, and tertiary amine groups capable of buffering the surrounding aqueous media at wide range of pH values (Tand and Szoka, 1997, Hyvönen et al., 2000). The mechanisms of PEI mediated DNA delivery in the cells suggest that the properties of PEI have impact on DNA escape from the endosomes (Ruponen et al., 2001), buffering of pH within the endosomes (Sonawane et al., 2003), endosomal swelling and lysis resulting from proton sponge effect (Sonawane et al., 2003), and interactions of PEI-DNA complexes with the glycosaminoglycans on the cell surface (Ruponen et al., 2004, Hanzliková et al., 2011). Also, some other polymers (e.g. polyamidoamine dendrimers, copolymers of 2-(dimethylamino) ethyl methacrylate based), and lipids (e.g. lipopolyamines) with secondary and tertiary amino groups show effective DNA delivery that is associated with endosomal buffering effects (Behr et al., 1989, Haensler and Szoka, 1993, Ruponen et al., 1999, Hyvönen et al., 2000). On the other hand, PLL contains only primary amines that all are protonated at physiological pH and thus has no extra buffering capacity for assisting endosomal escape (Tang and Szoka, 1997). Therefore, PLL polyplexes are not able to avoid trafficking to lysosomes and thus show low transfection efficiency (Akinc and Langer, 2002). Consequently, comparative structural investigations with PEI-DNA and PLL-DNA complexes may provide some general insights on the functionality of DNA complexes with cationic polymers and lipids.

Both PEI and PLL are capable to condense DNA into DNA-PEI nanoparticles stabilized by positive charges (Boussif et al., 1995, Ruponen et al., 1999). At N/P ratio of about 2 the PEI molecules completely neutralize DNA and the resulting polyplexes tend to aggregate easily (Van
de Wetering et al. 1998, Wagner, 2004), but addition of more PEI to this neutral core leads to the formation of a shell of extra PEI around the core. At that stage, the polyplexes become positively charged and aggregation is significantly reduced (Ikonen et al., 2008, Ketola et al., 2013). The same behaviour was shown for PLL polyplexes (Ketola et al., 2013). Previously, Kabanov et al. (Kabanov et al., 1998, Vinogradov et al., 1998) described the core-shell structure of DNA-polycation complexes. The core-shell structure of PEI polyplexes was further elaborated and two different stages of DNA were demonstrated with fluorescence analyses (Ketola et al., 2013).

Since many polyplexes deliver DNA only at high N/P ratios, it is evident that the core-shell structure has an important role in the non-viral gene delivery process.

Structure and function of PEI and other DNA complexes have been investigated using ethidium bromide as marker for the state of DNA (relaxed or condensed). Condensation of plasmid DNA (pDNA) has been routinely followed using quenching of fluorescence from ethidium bromide (Ruponen et al., 1999). More detailed analyses of the DNA state in the PEI and PLL polyplexes were carried out using time-resolved fluorescence, again with ethidium bromide as the reporter molecule (Ketola et al., 2013). However, the DNA condensing polymers may displace ethidium bromide from the complexes at N/P ratio of 2 and beyond (Vuorimaa et al., 2008, Banerjee et al., 2004, Ketola et al., 2011, Ketola et al., 2013) prohibiting the investigations of structural dynamics at N/P ratios above 2 (Fig. 1). The structural dynamics of PEI-DNA complexes at N/P ratios higher than 2 are important, because these polyplexes exert their highest gene transfer activity at high N/P ratios.

**Fig 1.** Behavior of ethidium bromide during polyplex formation: During the formation of polyplex core, ethidium bromide is squeezed out of the polyplex and thus the core-shell dynamics cannot be studied with it (Ketola et al., 2013).
Fluorescence resonance energy transfer (FRET) is a powerful spectroscopic tool in the investigations of short distance molecular interactions (Joo et al., 2008, Grecco and Verveer, 2011). In FRET process, the excitation energy is transferred from the donor fluorophore to the acceptor fluorophore by intermolecular long-range dipole–dipole interaction (Forester, 1946, Clegg, 1995, Breunig et al., 2006). Efficient energy transfer may take place when following requirements are met: 1) Emission spectrum of the donor and the absorption spectrum of the acceptor overlap, 2) Donor and acceptor molecules are in close proximity (1–10 nm), 3) Fluorescence quantum yield of the donor and the absorption coefficient of the acceptor are sufficiently high.

In this study, we utilized FRET to study the dynamics of the polymer:DNA complexes. Fluorophore pairs were selected to monitor the interactions between DNA and polymers (PEI, PLL) using steady state fluorescence spectroscopy (Vuorimaa et al., 2008, Ketola et al., 2011, Ketola et al., 2013). In contrast to the previous study with ethidium bromide, here we study polyplex behaviour at N/P ratios over two. Particularly, we prove that the shell PEI molecules are capable of moving and replacing the polymer molecules previously attached to DNA in polyplex core, while PLL is not. This feature explains the dynamic role of excess PEI in facilitating the gene transfer process in the cells and these principles may be applicable to many other DNA-polyplexes.

2. Materials and methods

2.1. Materials

The plasmid DNA (pCLuc4) encoding luciferase as a reporter gene with cytomegalovirus (CMV) viral promoter was grown in E. coli and was isolated and purified using a Qiagen Plasmid Giga kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The identity and purity of the plasmid were confirmed by agarose gel electrophoresis using restriction enzymes and by measuring absorbance at 260 and 280nm ($A_{260}/A_{280} > 1.8$), respectively. Branched polyethylenimine, PEI, (Sigma-Aldrich, St Louis, MO, USA) with a mean weight-average molecular weight of 25 kDa was purchased from Sigma-Aldrich used as a 1 mg/mL
aqueous solution adjusted to pH 7. Poly(L-lysine), PLL, with a mean molecular weight of 200 kDa (Sigma-Aldrich) was used in this study. For PLL the backbone amines are part of the peptide bonds. Thus, only the primary amines bind DNA and only they were taken into account when calculating the N/P ratios (the molar ratio of polymer nitrogen to DNA phosphate). For PEI the nitrogens of primary, secondary and tertiary amines were taken into account in the calculation of N/P ratios.

2.2. DNA, PLL and PEI labelling

PEI, PLL and DNA were labeled with fluorescent probes for the detection of the fluorescence changes caused by formation of the polyplexes at different N/P ratios. DNA was labeled with FITC (F) and both PEI and PLL with Cy3 (C) using Label It® Tracker Intracellular Nucleic Acid Localization Kits (Mirus Bio Corporation, Madison, WI, USA) following the manufacturer’s protocol. The labeling densities were 1.3 mol-% of bases for F-DNA, 0.2 and 0.6 mol-% of amines for C-PLL and C-PEI, respectively.

2.3. Sample preparation

All solutions were prepared in a buffer containing 50 mM MES, 50 mM HEPES, and 75 mM NaCl (adjusted to a pH 7.4 using 5 M NaOH). The final nucleotide concentration of DNA was 30 μM. The polyplexes were prepared stepwise: independent of the final N/P ratio between the cationic polymer and DNA, the initial solution with N/P = 0.6 was prepared by vigorous mixing of equal volumes of DNA and cationic polymer solutions. Emission spectrum of this initial solution was monitored to follow the complexation. After the measurement, the next N/P ratio was reached by adding appropriate amount of polymer solution. The measured N/P-values ranged from 0.6 to 6. The samples are denoted as “(DNA):(core polymer):(shell polymer)” with N/P-ratios as subscripts. For example the sample with FITC-labeled DNA, unlabeled PLL until N/P = 2 and Cy3-labeled PLL until the total N/P = 4 is described as F-DNA:PLL2:C-PLL2.
2.4. *Fluorescence measurements*

The design of the fluorescence measurements is shown in Fig. 2 and Table 1 shows the materials used in those experiments. In the experimental routes B and C, we utilized FRET with FITC-labeled DNA as the donor and Cy3-labeled polymers as acceptors to reveal the core-shell dynamics of the studied NPs.

![Diagram](image.png)

**Fig. 2.** Core dynamics studies: In route (A) and (B) the polyplex core was prepared from FITC-DNA and unlabeled polymer and the shell from either (A) unlabeled polymer or (B) Cy3-labeled polymer. In route (C) and (D) the polyplex core was prepared from FITC-DNA or DNA and Cy3-polymer and the shell either from (C) unlabeled polymer or (D) Cy3-polymer.

**Table 1.** Components of the *polyplexes*: DNA labeled with FITC (F-DNA), unlabeled DNA (DNA), unlabeled polymers, P (PLL and PEI), and polymers labeled with Cy3, C-P (C-PLL and C-PEI). The characters A-D refer to the logic shown in Figure 1.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Polymer</th>
<th>Core N/P = 2</th>
<th>Shell N/P ≥ 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>F-DNA</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>D</td>
<td>DNA</td>
<td>C-P</td>
<td>C-P</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>F-DNA</td>
<td>C-P</td>
<td>C-P</td>
</tr>
<tr>
<td>B</td>
<td>F-DNA</td>
<td>P</td>
<td>C-P</td>
</tr>
<tr>
<td>mixed <strong>B</strong></td>
<td>DNA</td>
<td>PEI</td>
<td>C-PLL</td>
</tr>
<tr>
<td>mixed <strong>C</strong></td>
<td>DNA</td>
<td>C-PLL</td>
<td>PEI</td>
</tr>
</tbody>
</table>
Steady-state emission and excitation spectra were recorded with a Fluorolog Yobin Yvon-SPEX fluorometer. The excitation wavelengths of 483 nm and 510 nm were used to excite the donor and the acceptor, respectively. The excitation spectra were monitored at 535 in the absence of Cy3 and at 610 nm in the presence of Cy3. The spectra were automatically corrected using a correction function provided by the manufacturer. The emission and excitation spectra were recorded twice immediately after preparation and at 0.5, 1.5 and 24 hours after preparation. The spectra were stable within the standard measurement error of the fluorometer. The results were analysed by the simple Stern-Volmer equation for dynamic quenching (Valeur, 2002)

\[
\frac{I_0}{I} = 1 + K_{SV}[Q]
\]

where \(I_0\) and \(I\) are the steady-state emission intensities at the donor maximum (520 nm) in the absence and presence of quencher, respectively, and \([Q]\) is the acceptor concentration. The Stern-Volmer constant \(K_{SV} = k_q \tau_0\), where \(k_q\) is the rate constant for quenching and \(\tau_0\) is the fluorescence lifetime of donor in the absence of acceptor.

3. Results

3.1. Properties of the labeled species

3.1.1. DNA labeled with FITC (F-DNA)

The emission and excitation spectra for F-DNA in the presence and absence of unlabeled PLL and PEI (Figure 2, route A) are shown in Fig. 3a. In the presence of PLL and PEI both spectra shift to red (Table 2) and the fluorescence efficiency decreases considerably up to N/P = 1.5 for PLL and N/P = 3 for PEI (Fig. 3b). During the formation of DNA polyplexes, the positive amine groups (N) of the cationic polymers bind the negative phosphate groups (P) of DNA. According to previous studies the P of DNA are completely bound by the N of the polymer at N/P ratios of 1.5 and 2.5 for PLL and PEI, respectively (Wagner, 2004, Hanzliková et al., 2011). The fluorescence quenching is due to the changes in the microenvironment of the FITC dye during the polycation complexation of DNA. Once the core is complete, further addition of polymer does not have any effect on the fluorescence of F-DNA.
Fig. 3. a) Excitation and emission spectra for F-DNA, F-DNA:PLL at N/P = 6 and F-DNA:PEI at N/P = 4. The excitation spectra were monitored at 535 nm, the emission spectra were excited at 483 nm. In all samples, the F-DNA concentration was constant corresponding to the 30 μM total DNA concentration. b) Area of the emission spectra for F-DNA:PLL and F-DNA:PEI at different N/P ratios.

Table 2. Maximum wavelengths of the absorption, excitation and emission spectra for the labeled species in the absence and presence of unlabeled polymers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>λ&lt;sub&gt;max, ABS&lt;/sub&gt; (nm)</th>
<th>λ&lt;sub&gt;max, EX&lt;/sub&gt; (nm)</th>
<th>λ&lt;sub&gt;max, EM&lt;/sub&gt; (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-DNA</td>
<td>-</td>
<td>495</td>
<td>517</td>
</tr>
<tr>
<td>F-DNA:PLL N/P = 6</td>
<td>-</td>
<td>499</td>
<td>522</td>
</tr>
<tr>
<td>F-DNA:PEI N/P = 6</td>
<td>-</td>
<td>498</td>
<td>527</td>
</tr>
<tr>
<td>C-PLL</td>
<td>560, 525</td>
<td>560, 525</td>
<td>572, 620</td>
</tr>
<tr>
<td></td>
<td>A&lt;sub&gt;525&lt;/sub&gt;/A&lt;sub&gt;560&lt;/sub&gt; = 0.55</td>
<td>I&lt;sub&gt;525&lt;/sub&gt;/I&lt;sub&gt;560&lt;/sub&gt; = 0.52</td>
<td></td>
</tr>
<tr>
<td>C-PEI</td>
<td>545, 510</td>
<td>545, 515</td>
<td>565</td>
</tr>
<tr>
<td></td>
<td>A&lt;sub&gt;510&lt;/sub&gt;/A&lt;sub&gt;545&lt;/sub&gt; = 0.93</td>
<td>A&lt;sub&gt;515&lt;/sub&gt;/A&lt;sub&gt;545&lt;/sub&gt; = 0.73</td>
<td></td>
</tr>
</tbody>
</table>

3.1.2 PLL and PEI labeled with Cy3 (C-PLL and C-PEI)

In the presence of unlabeled DNA the fluorescence properties of C-PLL depended on the N/P ratio. On addition of C-PLL to DNA in the amount corresponding to N/P = 0.6, the maxima of steady state emission and excitation spectra were red shifted 5 nm compared to the spectra without DNA. Then at low N/P ratios (N/P < 2), the core of the polyplex is forming and the spectra shifted back to blue and at N/P = 2 the spectra reach the same position as in the absence of DNA (Fig 4). At this point, the polyplex core is complete, C-PLL is not able to interact with DNA directly anymore, and on further addition of C-PLL the spectrum position does not change.
anymore. Thus, the fluorescence properties of C-PLL depend on whether it is in direct contact with DNA or not. Similar changes were observed also for C-PEI based polyplexes.

![Figure 4](image.png)

**Fig. 4.** a) Excitation and b) emission spectra for C-PLL in the presence of unlabeled DNA at N/P = 0.6, 2 and 6. The excitation spectra were monitored at 610 nm; the emission spectra were excited at 510 nm. The excitation spectrum maximum shifts from 565 nm to 560 nm and the emission spectrum maximum from 577 nm to 572 nm with increasing N/P ratio.

3.2. **Dynamics of polyplexes**

First the formation of the polyplex core and shell was studied using energy transfer between F-DNA as the donor and C-PLL or C-PEI as the acceptor (Fig. 2, route D). For PLL polyplexes (N/P = 0.6) the presence of F-DNA was seen as the absorption band at 490 nm and the emission band at 520 nm (Fig. 5a, b, solid lines). The presence of C-PLL was observed as the absorption bands at 565 and 530 nm as well as the emission bands at 578 and 625 nm. At N/P = 6, most of the F-DNA fluorescence was quenched and the fluorescence of C-PLL dominated the spectra (Fig. 5b, dotted line). Again, the absorption and emission bands of C-PLL shifted 5 nm towards the blue region at increasing N/P ratios (Fig. 5a, b). Similar behavior was observed also in PEI polyplexes.
Fig. 5. Normalized excitation a) and emission b) spectra for F-DNA:C-PLL system at N/P = 0.6 (solid lines) and 6 (dotted lines). Normalized excitation spectra for F-DNA:P₂ (solid lines), F-DNA:P₂:Cₚ₁ (dashed lines) and F-DNA:P₂:Cₚ₃ (dotted lines), where P is either c) PLL or d) PEI at N/P = 2, 3 and 6. Stern-Volmer plots for F-DNA:Pₖₐ₅₅₆₆₈ (squares), F-DNA:Cₚ₅₆₆₈ (circles) and F-DNA:P₂:Cₚ₃₄ (open diamonds), where P is either e) PLL or f) PEI. The FITC fluorescence intensity at 520 nm was used to calculate the intensity ratio $I_0/I$. The excitation spectra were monitored at 610 nm (acceptor emission) and the emission spectra were excited at 483 nm (donor excitation).

Next, we studied the exchange of polymers in the polyplex core and shell with the same donor and acceptor. For this purpose, polyplexes were prepared with unlabeled polymers until N/P = 2
ratios were reached (F-DNA:P₂). Thereafter, the N/P ratios were increased to 3 with Cy3-labeled polymer (F-DNA:P₂:C-P₁) followed by further additions of Cy3-labeled polymers up to N/P = 6 (F-DNA:P₂:C-P₄; Fig 2, route B). The excitation spectra for PLL and PEI at corresponding N/P ratios have been compared with each other in Fig. 5c, d. In the case of PLL the maximum wavelengths in the presence of Cy3 were independent on the N/P ratio and corresponded to the wavelengths observed for F-DNA:C-PLL₀,₆₋₆ system at N/P > 2 (Fig. 5c, dashed and dotted lines). Thus, the C-PLL does not interact directly with DNA and no PLL exchange takes place in the polyplex core. On the other hand, the blue spectral shift of F-DNA:PEI₂:C-PEI₁ samples after further addition of Cy3-labelled PEI up to N/P = 6 indicated a direct interaction between C-PEI and F-DNA (Fig. 5d, dashed and dotted lines). This suggests that PEI exchange takes place between the core and shell within the polyplex.

The full analysis of the fluorescence quenching for present systems of multivalent ligand binding to multi-subunit substrate is not straightforward. Furthermore, the changes in the microenvironment of the donor affect its fluorescence properties. Since the aim of this work is to study the dynamics of the polymer:DNA complexes, the simple Stern-Volmer plots (eq. 1) for F-DNA fluorescence quenching were used to compare the behavior of PEI and PLL polyplexes.

The plots for the quenching of F-DNA fluorescence in all studied systems namely F-DNA:P₀,₆₋₆, F-DNA:C-P₀,₆₋₆ and F-DNA:P₂:C-P₁₋₄ (Fig. 2, route A, B and D) are summarized in Fig. 5e and f. For F-DNA:PLL₀,₆₋₆ polyplex the curve was nearly linear indicating dynamic quenching (Fig. 5e, squares). Values for Kᵥ (0.15) and kₚ (3.94 × 10⁷ s⁻¹) were obtained using N/P ratios instead of acceptor concentration. For the F-DNA:C-PLL₀,₆₋₆ system Stern-Volmer curve deviated from linearity indicating that the fluorescence quenching was a mixture of dynamic and other quenching mechanisms (Fig. 5e, circles). The plot for F-DNA:PLL₂:C-PLL₁₋₄ polyplex followed the curve obtained for F-DNA:PLL₀,₆₋₄ suggesting that C-PLL in the polyplex shell did not have direct interaction with DNA in the core (Fig. 5e, open diamonds and squares). In the presence of PEI, none of the plots were linear, but the quenching for F-DNA:PEI₀,₆₋₆ system was much weaker than for F-DNA:C-PEI₀,₆₋₆ system (Fig. 5f, squares and circles). For F-DNA:PEI₂:C-PEI₁₋₄ system the plot followed that for F-DNA:PEI at N/P < 2 (Fig. 5f, open diamonds and squares). However, at higher N/Ps with C-PEI the quenching became more efficient indicating
that some of the shell C-PEI molecules reached the DNA and replaced the unlabelled PEI molecules in the polyplex core.

4. Discussion

According to the present results, the dynamics of the core and shell polymers in DNA polyplexes can be successfully studied using steady-state fluorescence spectroscopy with the used fluorescent probes. The FRET method and stepwise construction of different polyplexes presented herein allow similar investigations in any DNA polyplex system that can be labeled with appropriate fluorescent probes for FRET detection. We showed clear difference in the behavior of PEI and PLL. DNA-PEI polyplexes were capable of exchanging the PEI molecules between the core and shell, but such an exchange did not take place in DNA-PLL polyplexes. Although the present studies were performed in water solution, the observed dynamics between the core and shell should be an inherent property of polyplexes and relevant also in the actual gene transfer process in the cells.

Previously we showed by using ethidium bromide as a fluorescent marker, that the binding constant for formation of the polyplex core was 20% higher for PLL than for PEI (Ketola et al., 2013). This is relatively small difference and would not explain the large difference in DNA release and gene transfer efficacy between PLL and PEI polyplexes (Ruponen et al., 1999). DNA can be released from the PEI polyplexes upon competition by glycosaminoglycans, polyanions that are present on the cell surfaces and in the cells (Ruponen et al., 2001, Hanzliková et al., 2011). PLL polyplexes are quite inert in this respect and DNA release is at much lower levels than in the PEI polyplexes. Mobility of PEI between the core and shell of the polyplexes makes the PEI polyplexes more dynamic. It enables the exchange of PEI in the core of the polyplexes, leads to the DNA release in the cells and active transfection.

The effect of molecular structure on the polyplex formation includes the effects of: a) branching or side chains, b) the types of amines (primary, secondary tertiary) and their ratios, and c) the distribution of the amine groups i.e. amine density. Comparing the structures of branched PEI and PLL they differ in all three accounts. In PLL only the primary amines at the end of lysine
side chains do bind DNA and thus PLL has lower density of amine groups than PEI. However, the primary amine groups of PLL are all protonated at pH 7.4, whereas the degree of protonation of branched PEI’s variable amine groups is closer to 50% (Tang and Szoka, 1997). The average binding constants per amine are higher for PLL than for PEI (Ketola et al., 2013). Thus, the primary amine groups of PLL seem to bind DNA more strongly than the secondary amine groups of linear PEI and the combined affinity between the primary, secondary and tertiary amine groups of branched PEI (Ketola et al., 2013). This is in line with the observations indicating that the PEI–DNA complexes are more easily disrupted than PLL–DNA complexes in the presence of competing polyanions (Ruponen et al., 1999, Ruponen et al., 2001) and may explain the PEI exchange between the core and shell of polyplexes. Furthermore, in a recent study (Pigeon et al., 2016) it was found for polyplexes made from linear PEI and pDNA, the pDNA molecules are exchanged between the individual polyplexes. However, no such exchange was observed with polylysine polyplexes.

In the present study, the polymers of different molecular weights were selected in terms of their efficiency in DNA complexing and gene transfer. For instance, the smaller PLL (20 kDa) is not able to condense DNA at the same N/P ratios as the used PLL (200 kDa) and PEI (25 kDa) (Ruponen et al., 1999). Moreover, at physiological levels of salt the polyplexes formed with 20 kDa PLL are less soluble due to aggregation (Morille et al., 2008, Ward et al., 2001). Thus, the difference in molecular weights of the polymers used may also partially explain the reported findings. For the PLL the bulkier chain can limit the exchange of the polymer molecules in the polyplexes compared to the smaller PEIs.

DNA-complexing agents with only primary or quaternary amines (e.g. PLL, octa-arginine, DOTAP, DDAB) and the materials hosting also secondary and/or tertiary amines (e.g. PEI, DOGS, polyamidoamine dendrimers, polymers with histidines) have been shown to have distinct properties. Firstly, unlike primary amines, the secondary and tertiary amines are capable of binding hydrogen ions and buffering the pH changes in the endosomes (Kichler et al., 2001, Sonawane et al., 2003, Liang et al., 2012, Benjaminsen et al., 2013). When acidification of the endosomes is reduced, the DNA transfer is facilitated due to the decreased endosome–lysosomes fusion. Secondly, binding of the incoming endosomal hydrogen ions by the secondary
and tertiary amines leads to increased ion transport to the endosomes (also chloride ions), osmotic water flux, endosomal swelling and improved DNA escape to the cytosol (Sonawane et al., 2003). These events do not take place in the case of primary and quaternary amines. Thirdly, the polyplexes and lipoplexes with secondary and tertiary amines show increased DNA release from the NP carrier Ruponen et al., 2001, Hanzliková et al., 2011). The highest levels of transfection are reached at high N/P ratios if the carrier has secondary and tertiary amines involving complex interaction with the polyanionic cellular glycosaminoglycans (Hanzliková et al., 2011). These three factors are related to the PEI exchange between the core and shell, since PEI can accept and release hydrogen ions and bind/detach DNA molecules. Therefore, PEI polyplex is much more dynamic system than PLL polyplexes and this leads to favourable DNA transfection via several mechanisms.

5. Conclusions

In this study, we demonstrated that PEI molecules undergo exchange between the core and shell of the polyplexes. Reaching this conclusion required development of FRET methods and systematic construction of polyplexes with labeled and unlabeled DNA, PEI and PLL. This approach is broadly applicable in the investigations of the internal dynamics of various pharmaceutical nanoparticles. Structural dynamics of spontaneously assembling nanoparticles may give insights to the stability and functionality of the particles in drug delivery.

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Difference in the core-shell dynamics of polyethyleneimine and poly(L-lysine) DNA polyplexes.

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FIGURE LEGENDS

**Fig 1.** Behavior of ethidium bromide during polyplex formation: during the formation of polyplex core ethidium bromide is squeezed out of the polyplex and thus the core-shell dynamics cannot be studied with it (Ketola et al., 2013).

**Fig. 2.** Core dynamics studies: In route (A) and (B) the polyplex core was prepared from FITC-DNA and unlabeled polymer and the shell either from (A) unlabeled polymer or (B) Cy3-labeled polymer. In route (C) and (D) the polyplex core was prepared from FITC- DNA or DNA and Cy3-polymer and the shell either from (C) unlabeled polymer or (D) Cy3-polymer.

**Fig. 3.** a) Excitation and emission spectra for F-DNA, F-DNA:PLL at N/P = 6 and F-DNA:PEI at N/P = 4. The excitation spectra were monitored at 535 nm, the emission spectra were excited at 483 nm. In all samples, the F-DNA concentration was constant corresponding to the 30 μM total DNA concentration. b) Area of the emission spectra for F-DNA:PLL and F-DNA:PEI at different N/P ratios.

**Fig. 4.** a) Excitation and b) emission spectra for C-PLL in the presence of unlabeled DNA at N/P = 0.6, 2 and 6. The excitation spectra were monitored at 610 nm; the emission spectra were excited at 510 nm. The excitation spectrum maximum shifts from 565 nm to 560 nm and the emission spectrum maximum from 577 nm to 572 nm with increasing N/P ratio.

**Fig. 5.** Normalized excitation a) and emission b) spectra for F-DNA:C-PLL system at N/P = 0.6 (solid lines) and 6 (dotted lines). Normalized excitation spectra for F-DNA:P2 (solid lines), F-DNA:P2:C-P1 (dashed lines) and F-DNA:P2:C-P4 (dotted lines), where P is either c) PLL or d) PEI at N/P = 2, 3 and 6. Stern-Volmer plots for F-DNA:P0.6-6 (squares), F-DNA:C-P0.6-6 (circles) and F-DNA:P2:C-P1,4 (open diamonds), where P is either e) PLL or f) PEI. The FITC fluorescence intensity at 520 nm was used to calculate the intensity ratio I0/I. The excitation spectra were monitored at 610 nm (acceptor emission) and the emission spectra were excited at 483 nm (donor excitation).
Graphical abstract