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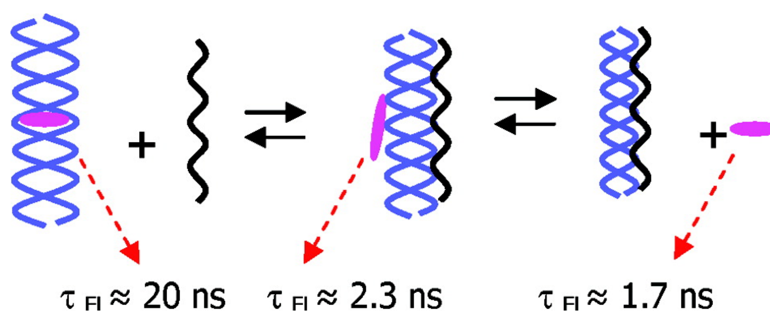

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Time-Resolved Fluorescence Spectroscopy Reveals Functional Differences of Cationic Polymer−DNA Complexes

Elina Vuorimaa,[†] Arto Urtti,[‡] Raimo Seppänen,[§] Helge Lemmetyinen,[†] and Marjo Yliperttula*,[||]

Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland, Centre for Drug Research and Division of Biopharmacy and Pharmacokinetics, University of Helsinki, Helsinki, Finland, and Department of Pharmaceutics, University of Kuopio, Finland

Received February 28, 2008; E-mail: marjo.yliperttula@helsinki.fi

Abstract: Cationic polymers bind DNA and form compacted nanoparticulates (i.e., polyplexes). Polyplexes augment DNA delivery into the cells as a nonviral method of gene therapy. DNA packing and release are the key factors in polyplex-mediated gene delivery, but they are poorly understood due to the lack of physical methods of investigation. We used time-resolved fluorescence spectroscopy to study poly(ethylenimine) (PEI) and poly(L-lysine) (PLL) polyplexes. Analysis of fluorescence lifetimes and time-resolved spectra revealed that DNA exists in several different states in PEI polyplexes and only in one tightly bound state in PLL polyplexes. The observed difference in the nature of the polyplexes may explain why PEI releases DNA more easily than PLL even though both polycations condense DNA effectively. The present method utilizing time-resolved fluorescence spectroscopy gives information on the specific interactions between DNA and the cationic polymers in the polyplexes. This kind of information is very important in the development of biologically effective nonviral systems for DNA delivery.

Introduction

Cationic polymers, peptides, and lipids are used to deliver DNA into cells as nanosized electrostatic complexes.[1] These nonviral methods avoid several problems that are associated with viral vectors, such as immunogenicity, generation of wild-type viruses, and inappropriate genomic integration. The relative lack of efficacy is the main limitation of nonviral gene transfer methods. There are several steps in successful gene transfer, including distribution to the right tissue, uptake into the target cells, escape of the material from the endosomes, diffusion in the cytoplasm, and access of DNA to the nucleus in transcription-active form.[1]

Release of DNA from the nanoparticulates is one of the key parameters. This was shown recently by Harashima and coworkers,[2] who showed that adenovirus vector was 8000 times more effective than liposomal vehicle, when the gene expression was normalized to the number of plasmid DNA copies in the nucleus. Similarly, Männistö et al.[3] showed a major difference between poly(ethylenimine) (PEI) and poly(L-lysine) (PLL) complexes with DNA. PEI polyplexes were 1−2 orders of magnitude more effective than PLL polyplexes even though similar numbers of plasmid DNA copies were delivered to the cell nuclei. Interestingly, DNA is released more easily from the PEI polyplexes than from PLL polyplexes if the DNA complexes are coincubated with biological polyanions, like glycosaminoglycans.[4] However, in both cases DNA is packed efficiently into small nanoparticulates. Controlled and triggered release of DNA, and siRNA, from the carrier complexes is essential for the successful development of nanocarriers for gene and gene silencing therapies.

Systematic design of polymeric and other cationic DNA delivery systems is hampered by the lack of structure−property relationships. DNA packing is poorly understood. This is due to the lack of accurate methods to study DNA complexes. Lamellar and hexagonal phases have been demonstrated in the case of cationic lipid−DNA complexes (lipoplexes), and toroidal and rod-shaped condensed structures are evident in the case of polyplexes.[5] Strikingly, there is a lack of understanding that would link physical polyplex properties with DNA release behavior. Such essential information is not available because there is a lack of feasible analytical techniques.

In this study, we developed fluorescence spectroscopic methods that enable analysis of the state of DNA in polyplexes. With this method we demonstrate that DNA in PEI polyplexes

[†] Tampere University of Technology.
[‡] Drug Discovery and Development Technology Center, University of Helsinki.
[§] University of Kuopio.
[||] Division of Biopharmacy and Pharmacokinetics, University of Helsinki.

(1) (a) Boussif, O.; Lezoualch, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297. (b) Friend, D. S.; Papahadjopoulos, D.; Debs, R. J. *Biochim. Biophys. Acta* **1996**, *1278*, 41. (c) Hyvönen, Z.; Ruponen, M.; Rönkkö, S.; Suhonen, P.; Urtti, A. *Eur. J. Pharm. Sci.* **2002**, *15*, 449.
(2) Hama, S.; Akita, H.; Ito, R.; Mizuguchi, H.; Hayakawa, T.; Harashima, H. *Mol. Ther.* **2006**, *13*, 786.
(3) Männistö, M.; Reinisalo, M.; Ruponen, M.; Honkakoski, P.; Tammi, M.; Urtti, A. *J. Gene Med.* **2007**, *9*, 479.
(4) Ruponen, M.; Ylä-Herttuala, S.; Urtti, A. *Biochem. Biophys. Acta* **1999**, *1415*, 331.
(5) Männistö, M.; Vanderkerken, S.; Toncheva, V.; Ruponen, M.; Elomaa, M.; Schacht, E. H.; Urtti, A. *J. Controlled Release* **2002**, *83*, 169.

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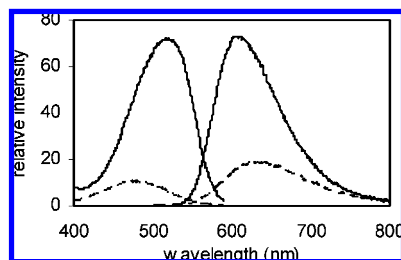


Figure 1. Fluorescence and excitation spectra of ETI in the absence (- - -) and presence (—) of DNA.

exists in several different states, whereas only one state was seen in the PLL polyplexes.

Materials and Methods

Materials. Plasmid DNA was pCMV-β that encodes β-galactosidase enzyme. The plasmid was produced in *Escherichia coli* and isolated and purified with a Qiagen kit. Branched poly(ethylenimine) (mean MW 22 000) and poly(L-lysine) (mean MW 200 000) were purchased from Sigma. These molecular weights were chosen on the basis of their activity as gene transfer agents.[4]

Sample Preparation. All solutions were prepared in 50 mM MES−HEPES buffer (pH 7.4). The final DNA concentration was adjusted to 300 μM per nucleotide, and the ethidium bromide (ETI): nucleotide ratio was 1:15. Independent of the final charge ratio between the cationic polymer and DNA, initially a solution with charge ratio (+/−)1:5 was prepared. In preparing this initial solution, the DNA/ETI solution and the cationic polymer solution were mixed at a volume ratio of 1:1. Solutions were mixed rigorously to ensure effective complexation between DNA and the polymer. Complexation was followed by measuring the fluorescence spectrum of the initial solution. After this procedure the final charge ratio was adjusted by appropriate addition of the polymer solution.

Fluorescence Measurements. Steady-state fluorescence and excitation spectra were recorded with a Fluorolog-3 (Spex Inc.) fluorometer. The excitation wavelength was 480 nm and the spectra were corrected by applying a correction function supplied by the manufacturer. The relative fluorescence quantum yield for DNA/ETI and free ETI were calculated from the steady state spectra by dividing the spectral areas by the absorbance at the excitation wavelength and normalizing the results to give $\Phi_{DNA/ETI}^{rel} = 1$. Time-resolved fluorescence was measured by the time-correlated single-photon counting technique. The excitation wavelength was 340 nm and the time resolution was about 300 ps. To diminish the influence of the scattered excitation, a cutoff filter was used in front of the monitoring monochromator. To study time-resolved spectra, the decays were collected with a constant accumulation time (30 min) in the 550−670 nm wavelength range with steps of 20 nm. The decays were simultaneously fitted to the sum of exponents:

$$I(t, \lambda) = \sum a_i(\lambda) e^{-t/\tau_i} \quad (1)$$

where τ_i is the global lifetime and $a_i(\lambda)$ is the local pre-exponential factor at a particular wavelength. The factors $a_i(\lambda)$ represent the decay-associated spectra (DAS), which in the case of a mixture of different noninteracting fluorescing species correspond to the individual spectra of the species. The spectral areas (A_i) of the components can be calculated by integrating the pre-exponential factors over the measured wavelength range. Taking into to account the relative fluorescence quantum yields of the species Φ_i^{rel} leads to

$$A_i = \Phi_i^{rel} \int a_i(\lambda)\, d\lambda \quad (2)$$

Results

Steady-State Fluorescence. Fluorescence and excitation spectra of DNA/ETI complex and free ETI in buffer (pH = 7.4) are shown in Figure 1. In the presence of DNA, the fluorescence quantum yield of ETI increases due to intercalation with DNA: the relative fluorescence quantum yield of free ETI, Φ_{ETI}^{rel}, is 0.17. The increase in fluorescence intensity is accompanied by a hypsochromic shift in the fluorescence maximum from 630 nm in the absence of DNA to 610 nm in its presence. The absorption spectrum also changes: the absorption maximum shifts from 480 nm in the absence of DNA to 520 nm in its presence.

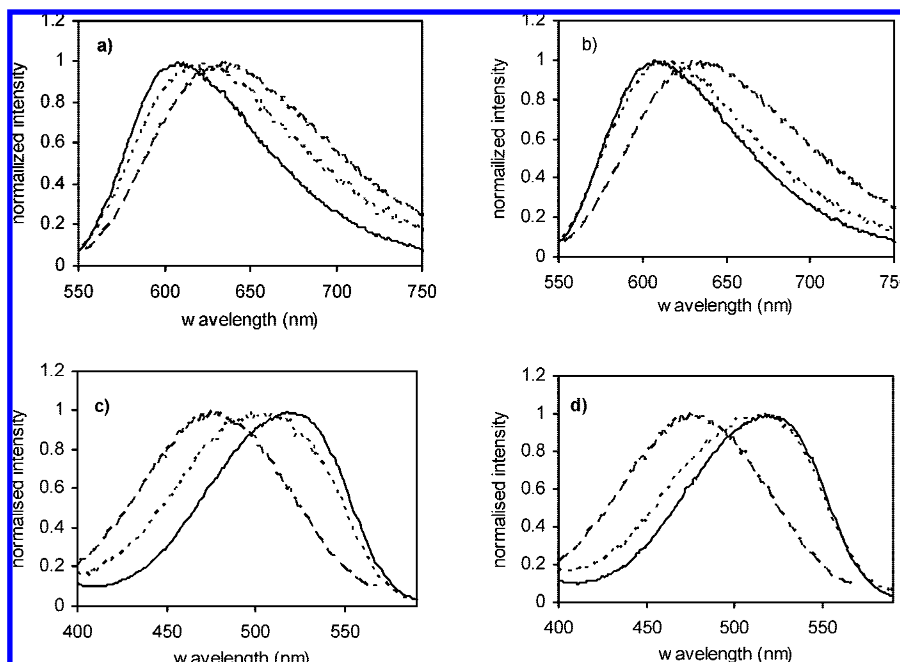


Figure 2. Normalized fluorescence (a, b) and excitation (c, d) spectra of ETI/DNA complexes in the presence of different amounts of PEI (a, c) or PLL (b, d). (—) Polymer:(ETI/DNA) = 0:1; (•••) polymer:(ETI/DNA) = 2.6:1; (- - -) ETI in the absence of DNA.

Table 1. Ratios of Bound ETI and Free ETI (*ED*/*E*) Obtained from Steady-State Fluorescence Spectra

sample and charge ratio	*ED*/*E*
PEI25:(ETI/DNA) = 0.2:1	*a*
PEI25:(ETI/DNA) = 0.4:1	1.224 712
PEI25:(ETI/DNA) = 0.7:1	0.367 918
PEI25:(ETI/DNA) = 1:1	0.204 119
PEI25:(ETI/DNA) = 1.3:1	0.222 024
PEI25:(ETI/DNA) = 1.6:1	0.180 384
PEI25:(ETI/DNA) = 2.6:1	0.136 079
PEI25:(ETI/DNA) = 5.2:1	0.125 611
PLL:(ETI/DNA) = 0.2:1	*a*
PLL:(ETI/DNA) = 0.4:1	0.455 569
PLL:(ETI/DNA) = 0.7:1	0.397 564
PLL:(ETI/DNA) = 1.3:1	0.349 918
PLL:(ETI/DNA) = 2.6:1	0.317 518

[a] At this charge ratio the polymer did not change the spectrum of DNA.

Upon addition of cationic polymers (PEI or PLL) into the ETI/DNA solution, complexation between DNA and the polymer takes place. This complexation at a ratio of +/−3:1 induces a conformation change in the DNA that squeezes some of the ETI molecules from the DNA to the solution. This is seen in the fluorescence spectra as a polymer-concentration-dependent decrease in the ETI fluorescence intensity and a blue shift of the fluorescence maximum. The normalized fluorescence spectra at different charge ratios are shown in Figure 2. For PEI the blue shift is more pronounced than for PLL. At charge ratio 2.6:1 the fluorescence maximum in the presence of PEI25 is at 620 nm, whereas in the presence of PLL it is at 615 nm. A similar change, but in the opposite direction, is observed in the excitation spectra. For PEI the change is clear, but for PLL the change is very small (Figure 2).

Unfortunately, only the solutions with charge ratio polymer:DNA = 0.2:1 were clear; at higher charge ratios the solutions were more or less cloudy. Thus, the fluorescence intensities could not be used to monitor the complexation between DNA and polymer, and the shift in the fluorescence maximum was used instead. Normalized fluorescence spectra at different charge ratios were fitted with a linear combination of fluorescence spectra of ETI in the absence and presence of DNA. By use of the areas of the fitted component spectra and taking into account the relative fluorescence efficiencies of the species, a ratio *ED*/*E* is obtained. The obtained values are listed in Table 1. For the smallest charge ratio, polymer:(ETI/DNA) = 0.2:1, the shapes of the spectra were identical with that in the absence of the polymers. At higher charge ratios the *ED*/*E* ratio decreases with increasing charge ratio for both polymers, but for PEI the decrease is larger than for PLL.

Time-Resolved Fluorescence. Due to complexation with DNA, the fluorescence lifetime of ETI increases from 1.7 ± 0.1 ns in buffer to 21.4 ± 0.1 ns in the presence of DNA. With the DNA:ETI molar ratio used, ETI is fully bound by DNA, which is observed as a one-exponential time decay in the presence of DNA.

In the presence of the cationic polymers at charge ratios higher than 1:5, two-exponential fluorescence decays were observed (Figure 3). The decay curves were fitted with a two-exponential decay model, and the obtained lifetimes are listed in Table 2.

In the presence of PLL200, the fluorescence lifetimes are independent of the charge ratio and are in good agreement with the lifetimes measured for free ETI and ETI/DNA complex. However, in the presence of PEI25, at low charge ratios (under 0.7:1), the lifetime of the short-lived component is higher, 2.3 ± 0.4 ns, than at higher charge ratios (over 0.7:1). The longer fluorescence lifetime is fluctuating in the case of PEI polyplexes (from 17.5 to 22.5 ns). For both polymers the A_2/A_1 ratio decreases with increasing charge ratio, but for PEI the decrease is stronger than for PLL.

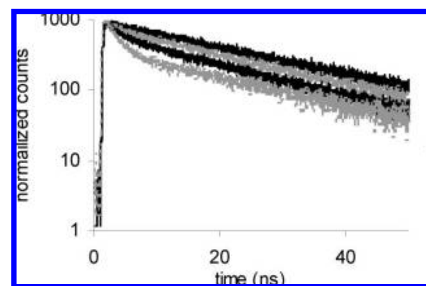


Figure 3. Fluorescence decay curves of ETI/DNA complex in the presence of different amounts of PEI: (1) PEI:(ETI/DNA) = 0:1, (2) PEI:(ETI/DNA) = 0.4:1, (3) PEI:(ETI/DNA) = 0.7:1, and (4) PEI:(ETI/DNA) = 2.6:1. The excitation wavelength was 340 nm and the fluorescence was monitored at 610 nm.

Table 2. Fluorescence Lifetimes (τ_i) Obtained with Two-Exponential Fit of the Fluorescence Decay Curves

sample and charge ratio	τ_1 (ns)	τ_2 (ns)	A_2/A_1 [a]
ETI	1.7 ± 0.1		
ETI/DNA		21.4 ± 0.1	
PEI25:(ETI/DNA) = 0.2:1		21.4 ± 0.4	
PEI25:(ETI/DNA) = 0.4:1	2.3 ± 0.4	19.9 ± 1.8	0.441 817
PEI25:(ETI/DNA) = 0.7:1	2.3 ± 0.4	19.5 ± 1.7	0.123 86
PEI25:(ETI/DNA) = 1:1	1.7 ± 0.5	17.5 ± 1.3	0.121 672
PEI25:(ETI/DNA) = 1.3:1	1.7 ± 0.2	21.4 ± 0.8	0.139 911
PEI25:(ETI/DNA) = 1.6:1	1.8 ± 0.2	21.4 ± 0.8	0.112 673
PEI25:(ETI/DNA) = 2.6:1	1.9 ± 0.1	19.0 ± 0.3	0.101 859
PEI25:(ETI/DNA) = 5.2:1	1.8 ± 0.2	22.5 ± 1.5	0.084 889
PLL:(ETI/DNA) = 0.2:1	1.7 ± 0.2	20.2 ± 0.4	0.446 186
PLL:(ETI/DNA) = 0.4:1	1.7 ± 0.2	20.4 ± 0.5	0.331 537
PLL:(ETI/DNA) = 0.7:1	1.7 ± 0.3	20.2 ± 0.8	0.270 103
PLL:(ETI/DNA) = 1.3:1	1.8 ± 0.4	20.3 ± 0.4	0.239 866

[a] The ratio of the spectral areas of the components, A_2/A_1, was calculated according to eq 2.

Time-resolved spectra in the presence of the polymers at different charge ratios are shown in Figure 4. At low charge ratios, under 1:1, the spectra remain nearly constant over time for both polymers. At higher charge ratio (4:1) the time-resolved spectra of PEI and PLL polyplexes are different: PEI shows broadened half-width and a much clearer blue shift in the spectra than PLL.

Discussion

The fluorescence measurements were carried out once the binding between the polymers and DNA had already reached equilibrium. Since ETI is the only fluorescing component in the sample, we use its fluorescence (FL) to monitor the equilibrium according to Scheme 1. The asterisk indicates an electronically excited state of the species, and P is a cationic polymer. For the complexation with PLL this scheme seems to fit very well: the lifetimes of the components stay constant independent of the charge ratio. The longer component of about 20 ns corresponds to [DNA/ETI] complex and the shorter 1.7 ns component corresponds to free ETI.

In the presence of PEI this simple scheme is inadequate: at low charge ratios, the behavior of the fluorescence signals does not fit the scheme. Thus, we propose a two-step scheme in the presence of PEI (Scheme 2). In the first step, DNA/polymer

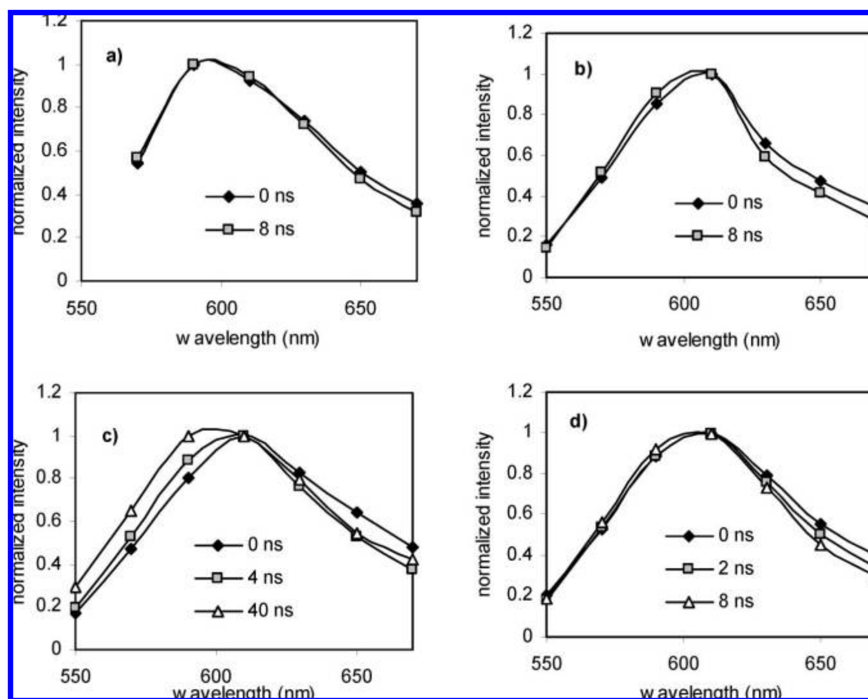
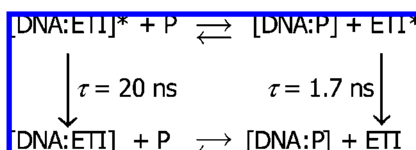
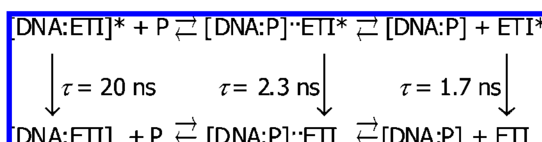


Figure 4. Time-resolved spectra of ETI/DNA complex at different times after excitation: (a) PEI:(ETI/DNA) = 0.4:1; (b) PLL:(ETI/DNA) = 0.4:1; (c) PEI:(ETI/DNA) = 2.6:1; (d) PLL:(ETI/DNA) = 2.6:1.

Scheme 1



Scheme 2



complex is formed. However, part of ETI is not released to the bulk solution but remains loosely bound to the DNA/polymer complex. This leads to the fluorescence lifetime of 2.3 ns, which is remarkably shorter than for the DNA/ETI complex but still longer than 1.7 ns lifetime of ETI in bulk solution. At higher charge ratio, the remaining ETI is released to the bulk solution. Since the fluorescence lifetimes of the loosely bound ETI and free ETI are so close to each other, already at charge ratio 0.7:1

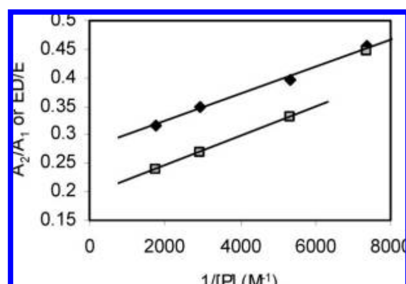


Figure 5. Ratios A_2/A_1 (□)and *ED/E* (◆) as a function of PLL concentration (as mol charges dm^{-3}).

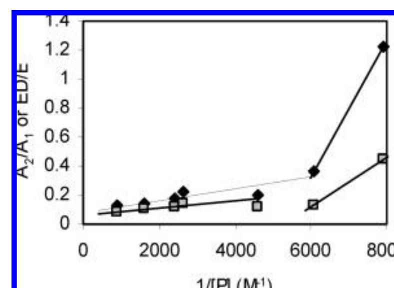


Figure 6. Ratios A_2/A_1 (□) and *ED/E* (◆) as a function of PEI concentration (as mol charges dm^{-3}).

the fluorescence of ETI in bulk solution is dominating the decay curves at short times. Thus, in addition to the long-lived component of ETI/DNA complex, only the 1.7 ns component is resolved in the fitting. The proposed scheme also explains the differences observed in time-resolved spectra at low charge ratios: since ETI is loosely bound to the DNA/PEI25 complex, the fluorescence maximum stays at shorter wavelengths.

The previous experiments suggest that, unlike PLL polyplexes, the PEI polyplexes are easily relaxed and disintegrated in the presence of polyanions, even at higher charge ratios like 2.6:1.[6] These effects were not affected by the molecular weight of PEI and PLL. Time-resolved spectroscopy shows interesting differences for the bound DNA among PEI and PLL polyplexes. Both time-resolved spectra (Figure 4) and the ∼20 ns component of the fluorescence decay curves (Table 1) support the notion that DNA exists in several states in the PEI polyplexes but not in PLL polyplexes. Increased half-width of the time-resolved spectra and the fluctuating values of the lifetimes indicate that DNA is not folded into a fixed conformation but rather it has several conformations, which may be changing continuously over time.

(6) Itaka, K.; Harada, A.; Yamasaki, Y.; Nakamura, K.; Kawaguchi, H.; Kataoka, K. *J. Gene Med.* **2004**, *6*, 76.

Binding Constant in the Case of PLL. ETI does not play a role in the electrostatic complexation reaction of the polymer and DNA. Rather, it is a fluorescent probe for monitoring the equilibrium between DNA and the cationic polymers.[7] We are monitoring the equilibrium

$$[\mathrm{DNA/ETI}] \rightleftarrows \mathrm{DNA} + \mathrm{ETI} \quad (3)$$

which takes place between a small molecule, ETI, and a macromolecule, DNA. Thus the formulation of this type of situation can be used in determining the binding between two macromolecules:

$$\mathrm{DNA_E} + \mathrm{P} \rightleftarrows [\mathrm{DNA/P}] \quad (4)$$

where $\mathrm{DNA_E} = [\mathrm{DNA/ETI}]$. The proportion of DNA bound by the polymer, B, is given by

$$B = \frac{[\mathrm{DNA/P}]}{[\mathrm{DNA_E}] + [\mathrm{DNA/P}]} \quad (5)$$

When this is combined with the equilibrium constant of eq 4, we obtain

$$B = \frac{K[\mathrm{P}][\mathrm{DNA_E}]}{[\mathrm{DNA_E}] + K[\mathrm{P}][\mathrm{DNA_E}]} = \frac{K[\mathrm{P}]}{1 + K[\mathrm{P}]} \quad (6)$$

which can be presented in a linear form as $1/B = 1 + 1/K[\mathrm{P}]$. To correlate the factor B with time-resolved fluorescence measurements, the spectral areas of the components, A_i, were used. A_1 values obtained for the short-lived component correspond to [DNA/P], and A_2 values obtained for the long-lived component correspond to $[\mathrm{DNA_E}]$. Thus, the ratio B can be expressed as $B = A_1/(A_2 + A_1) \propto A_1/A_2$ and eq 6 becomes

$$\frac{A_2}{A_1} = C + \frac{1}{K[\mathrm{P}]} \quad (7)$$

where C is a proportionality factor correcting the difference between the spectroscopic data and actual concentration. Thus, plotting the ratio A_2/A_1 as a function of inverse PLL concentration (as mol charges $\mathrm{dm^{-3}}$) we should get a linear dependence with the binding constant equal to the inverse of the slope. By similar reasoning for the ED/E ratio obtained from the steady-state fluorescent measurements, we get

$$\frac{ED}{E} = C' + \frac{1}{K[\mathrm{P}]} \quad (8)$$

In Figure 5 the ratios A_2/A_1 and ED/E are plotted as a function of PLL concentration. When the data for the lowest charge ratio (0.4) for time-resolved measurements is discarded, the binding constants obtained from the two measurements are nearly equal being 39 000 mol charges $\mathrm{dm^{-3}}$ and 42 000 mol charges $\mathrm{dm^{-3}}$ from time-resolved and steady-state measurements, respectively.

Binding Constant in the Case of PEI25. According to Scheme 2, the complexation is now a two-step reaction which can be simplified to the form

$$[\mathrm{DNA_E}] + \mathrm{P} \rightleftarrows [\mathrm{DNA_E/P}] \rightleftarrows [\mathrm{DNA/P}] \quad (9)$$

where the subscript E in DNA emphasizes that ETI is no longer tightly intercalated in the DNA but is still loosely bound to it. Only in the last step is ETI completely released into the solution. The equilibrium constant for this system can be expressed as

$$K_{\mathrm{tot}} = K_1 K_2 = \frac{[\mathrm{DNA_E/P}]}{[\mathrm{DNA_E}][\mathrm{P}]}\frac{[\mathrm{DNA/P}]}{[\mathrm{DNA_E/P}]} = \frac{[\mathrm{DNA/P}]}{[\mathrm{DNA_E}][\mathrm{P}]} \quad (10)$$

At high PEI concentration B is given by

$$B = \frac{[\mathrm{DNA/P}]}{[\mathrm{DNA_E}] + [\mathrm{DNA/P}]} = \frac{K_2[\mathrm{P}]}{1 + K_2[\mathrm{P}]} \quad (11)$$

On the other hand, at low charge ratios the second step is negligible and the equilibrium constant is

$$K_1 = \frac{[\mathrm{DNA_E/P}]}{[\mathrm{DNA_E}][\mathrm{P}]} \quad (12)$$

and the average number of sites occupied by the polymer B is given by

$$B = \frac{[\mathrm{DNA_E/P}]}{[\mathrm{DNA_E}] + [\mathrm{DNA_E/P}]} = \frac{K_1[\mathrm{P}]}{1 + K_1[\mathrm{P}]} \quad (13)$$

In both cases the equation has the same form and turning to a linear form gives eq 6. By use of the same relations as in the case of PLL (eqs 7 and 8) the ratios A_2/A_1 and ED/E are plotted as a function of PEI concentration in Figure 6. At high PEI concentrations the binding constants K_{tot} obtained from the two measurements are in relatively good agreement, being 38 000 and 20 000 M/charge from time-resolved and steady-state measurements, respectively. At low PEI concentrations the relative difference is much larger, yielding binding constants K_1 of 6000 and 2000 mol charges $\mathrm{dm^{-3}}$ from time-resolved and steady-state measurements, respectively. This disagreement is probably due to the presence of the complex where ETI is loosely bound to the DNA/polymer complex. The shape of the fluorescence spectrum of this species is unknown as is its relative fluorescence quantum yield. According to the time-resolved spectra, it seems that the shape is close to that of ETI/DNA. However, in this complex ETI is more exposed to the solvent than when it is intercalated in DNA. Thus, its fluorescence quantum yield is probably much lower. This discrepancy leads to overestimation of the proportion of the tightly bound ETI/DNA complex in the ED/E ratio obtained from the steady-state fluorescence spectra. Similar error does not occur in the A_2/A_1 ratio, since the lifetime of the loosely bound complex differs so clearly from that of the tightly bound complex.

Relevance in DNA Delivery. It is known from previous studies that PEI polyplexes are easily relaxed and eventually may release DNA in the presence of biological polyanions, such as heparan sulfate and chondroitin sulfate.[4] On the contrary, PLL polyplexes are resistant against competition by polyanions. This is not related to the packing density of the complexes. In fact, PEI polyplexes have smaller diameter than PLL polyplexes,[3] and also this study shows higher DNA condensation with PEI compared to PLL (Table 1). It seems that, despite efficient DNA packing, PEI is able to release DNA more easily than PLL. This may be important in gene delivery: the transfection activity from PEI polyplexes was much higher than from PLL polyplexes.[4]

The fluorescence spectroscopic measurements of this study show a clear difference in the profiles of PEI and PLL polyplexes. There are two major differences. First, upon complexation at low ± charge ratios for PEI polyplexes, an intermediate state between tightly bound and free DNA with fluorescence lifetime of 2.3 ns was observed. This state was not oberved for the PLL polyplexes. Second, for the free DNA

(7) LePecq, J. B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.

only one distinct state was observed in the presence of PLL, but in the presence of PEI the free DNA seems to exist in several slightly different states. This is a fundamental difference seen in two sets of data. The lifetimes of longer-lived component in the fluorescence decay curves show fluctuation in the PEI data (17.5−22.5 ns) but not in the case of PLL (20.2−20.4 ns) (Table 1). In addition, the time-resolved spectra of PEI polyplexes show a multitude of spectra and broad half-width, but the profile of PLL does not show these features (Figure 4). Such a range of different states of DNA in the PEI polyplexes may be due to the fact that PEI is a branched polymer and has primary, secondary, and tertiary amines, while PLL has only primary amines.[1] Binding by PLL seems to result in one bound and stable state of DNA: these complexes cannot be disintegrated by competing polyanions.[4] The various bound states of PEI may have different binding energies and these states may undergo continuous changes between localized equilibria. Such complexes may release DNA more easily, which is a desirable property in DNA delivery, if the release can be limited to the right intracellular site.

Previously, DNA binding with polycations has been investigated only by steady-state fluorescence spectroscopy[8] but not by time-resolved spectroscopy. The time-resolved method turned out to be useful in finding out differences between PLL and PEI polyplexes. This kind of method is needed in order to understand the functionality of the complexes between gene medicines (DNA, siRNA, antisense) and cationic carriers (polymers, lipids, peptides).

(8) Zelikin, A. N.; Trukhanova, E.; Putnam, D.; Izumrudov, V. A.; Litmanovich, A. A. *J. Am. Chem. Soc.* **2003**, *125*, 13693.

Conclusions

The obtained results show that time-resolved fluorescence measurements are useful in characterizing the binding of DNA with polycations. Analysis shows that DNA complexed with PEI has several different bound states and no single locked conformation, whereas the PLL polyplexes have DNA in only one conformation. This approach may be useful in understanding and characterizing the physical-chemical features of nonviral delivery systems for gene medicines.

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