Monitoring of the Formation and Dissociation of Polyethylenimine/DNA Complexes by Two Photon Fluorescence Correlation Spectroscopy

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ABSTRACT Polyethylenimines (PEI) constitute efficient nonviral vectors for gene transfer. However, because free PEI shows some cytotoxicity and because intracellular dissociation of PEI/DNA complexes seems to be required for efficient transfection, it is important to monitor the concentrations of free and bound partners in the mixtures of DNA and PEI used for transfection. To reach this objective, we used fluorescence correlation spectroscopy with two-photon excitation to characterize the complexes formed with either rhodamine-labeled 25 kDa PEI or DNA plasmid molecules. At the molar ratios of PEI nitrogen atoms to DNA phosphate usually used for transfection, we found that ~86% of the PEI molecules were in a free form. The PEI/DNA complexes are composed on the average by 3.5 (±1) DNA plasmids and ~30 PEI molecules. From this composition and the pK_a of PEI, it could be inferred that in contrast to DNA condensation by small multivalent cations, only a limited neutralization of the DNA phosphate groups is required for DNA condensation by PEI. Moreover, DNA appears only poorly compacted in the PEI/DNA complexes. As an application, fluorescence correlation spectroscopy was used to monitor the purification of PEI/DNA complexes by ultrafiltration as well as the heparin-induced dissociation of the complexes.

INTRODUCTION

Gene therapy focuses on the therapeutic use of genes for the treatment of several important diseases. However, due to their size and charge, nucleic acids hardly cross cell membranes. As a consequence, the success of gene therapy strongly depends on the use of vectors that could efficiently deliver the therapeutically active genes into the cells. Though viral vectors are highly efficient for this purpose, numerous efforts are done to develop synthetic vectors because they are less immunogenic and pathogenic.

Among the nonviral vectors, the polyethylenimine (PEI) class shows promising results for gene delivery, both in vitro and in vivo (Boussif et al., 1995; Godbey et al., 1999b; Remy et al., 1998). PEI are polymeric molecules that exist either as a linear or a branched form. Because every third atom of the PEI backbone is an amino nitrogen, they exhibit a high cationic charge density potential (Boussif et al., 1995) and considerable buffer capacity (Suh et al., 1997; Tang and Szoka, 1997; von Harpe et al., 2000). The transfection efficiency of PEI depends, in a still ill-defined way, on their average molecular weight and polydispersity (Godbey et al., 2000; Godbey et al., 1999a; Godbey et al., 1999b; Kichler et al., 1999; Remy et al., 1998). Moreover, to be efficiently internalized and optimally diffuse in vivo, the PEI/DNA complexes need to be of small size (<100 nm). This is usually achieved by adjusting the molar ratio of PEI nitrogen atoms to DNA phosphate (N/P) between six and 10 (Boussif et al., 1995; Finsinger et al., 2000; Ogris et al., 1998). These conditions lead to particles with strong positive surface charge that induces repulsion interactions among the particles and prevents aggregation. However, this protocol also generates significant amounts of free PEI (Finsinger et al., 2000) that turns out to be toxic for the cell (Fischer et al., 1999; Godbey and Mikos, 2001; Godbey et al., 2001; Putnam and Langer, 1999).

The intracellular path of PEI/DNA complexes has been recently characterized in two different cell lines (Godbey et al., 1999c; Remy-Kristensen et al., 2001). In both cases, the complexes have been shown to be internalized by endocytosis. Subsequently, the complexes accumulate in late endosomes and lysozomes around the nucleus. Only a limited number of complexes reach the nucleus. Both the escape from endosomes as well as the transport to the nucleus are still largely unknown. Additionally, the critical question of the dissociation of the complexes during these steps is also unsolved. As a consequence, to further understand the mechanism of PEI-mediated transfection and rationally improve the protocol of transfection, it would be important to determine the composition of PEI/DNA complexes and monitor the composition changes in the cell. Fluorescence correlation spectroscopy (FCS) associated with two-photon excitation (TPE) seems well-suited for this purpose. Indeed, FCS has been shown to be useful for monitoring and determining the kinetic and thermodynamic parameters of various interactions both in test tubes and cells (for a review, see Hess et al. (2002)). Moreover, FCS has been recently used to monitor the in vitro condensation of DNA by various nonviral vectors (Kral et al., 2002a; Kral et al., 2002b; Van Rompaey et al., 2001). In this context, the aim of the present work was to investigate by FCS the interaction of DNA with a branched PEI of 25 kDa reported to give a high transfer efficiency (Godbey et al., 1999b; Kichler et al., 1999; Remy et al., 1998). This allowed us to determine the concentrations of free and bound partners, and to deduce the stoichiometry of the complexes. The changes in the concentrations of the

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partners were further evaluated after an ultrafiltration procedure that has been reported to increase transfection efficiency (Finsinger et al., 2000), and after addition of heparin that has been shown to dissociate the PEI/DNA complexes (Moret et al., 2001). TPE was chosen because of its intrinsic limitation of the volume sample as well as its good signal to noise ratio (Schwille et al., 1999).

MATERIALS AND METHODS

Chemicals

5-Carboxytetramethylrhodamine (TMR) was purchased from Molecular Probes (Leiden, The Netherlands). Nonlabeled PEI was from Aldrich (St. Quentin, France). TRITC (tetramethylrhodamine isothiocyanate)-labeled PEI (25 kDa) was a kind gift from J. P. Behr (Illkirch, France). It was determined that ~1% of the amines are labeled in TRITC-labeled PEI molecules. This corresponds to an average of six fluorophores for a 25 kDa PEI molecule. The buffer was 20 mM Hepes, pH 7.4. The pCMV-Luc plasmid (5.8 kbp) coding for *Photinus pyralis* luciferase was amplified by standard molecular biology techniques, using a Jetstar plasmid purification kit (Genomed, Bad Oeynhausen, Germany) as previously described (Remy-Kristensen et al., 2001). The rhodamine-labeled pGeneGrip plasmid (5.1 kbp) was purchased from Gene Therapy Systems (San Diego, CA). The plasmids were homogeneously labeled with ~5–8 fluorophores per plasmid. Concentrations of DNA stock solutions were determined at 260 nm on a Cary 400 spectrophotometer and the plasmid integrity was checked by gel electrophoresis. Heparin (mol wt 9 kDa) was kindly provided by C. Boudier (Illkirch, France).

Preparation of PEI/DNA complexes

PEI/DNA complexes were prepared as described (Boussif et al., 1995). Briefly, equal volumes of 160, 240, or 400 μM (expressed in amine groups) PEI solution and 40 μM (expressed in phosphate groups) DNA were mixed to reach a 20-μM final concentration of DNA and a nitrogen per phosphate (N/P) ratio of respectively 4, 6, or 10. Solutions were then briefly vortexed and left for equilibration for a minimum time of 10 min (Boussif et al., 1995) before performing FCS measurements.

Ultrafiltration of PEI/DNA complexes

Two milliliters of PEI/DNA complexes with a final concentration of 100 μM DNA were filtered as described (Finsinger et al., 2000) on Centricon-100 membranes (Millipore, Saint-Quentin, France). Five rounds of five minutes centrifugation at 250 $\times$ *g* were performed on a Sigma 4K15 centrifuge with swinging buckets. Between each round, the volume of solution was re-adjusted to 2 ml with buffer. After filtration, complexes were collected from the solution above the filter and rediluted in buffer.

Fluorescence correlation spectroscopy

FCS measurements were performed on a home-build setup (Fig. 1). TPE is provided by a Tsunami Ti:Sapphire laser pumped with a Millennia V solid-state laser (Spectra-Physics, Mountain View, CA). Pulses of ≈100 fs are produced with an 80-MHz frequency at an 850-nm wavelength. After a beam expander, the infrared light is focused into the sample by a water immersion Olympus objective (60×, NA = 1.2) mounted on an Olympus IX70 inverted microscope. The back aperture of the objective is slightly overfilled, creating a diffraction-limited focal spot. Samples are placed in eight wells Lab-Tek chambered cover glass (Nalge Nunc International, Rochester, NY) positioned in the *X* and *Y* directions by a motorized stage (Märzhäuser, Germany). The fluorescence signal from the samples is collected through the same objective and directed by a COWL750 dichroic mirror (Coherent, Orsay, France) toward a 50-μm diameter optical fiber coupled to an avalanche photodiode (SPCM 200 FC, EG&G, Canada). The residual infrared light is rejected by a BG39 Filter (Coherent). The normalized autocorrelation function (ACF), $G(\tau)$, of the fluorescence intensity fluctuations is calculated online by an ALV5000E digital correlator card (ALV, Langen, Germany).

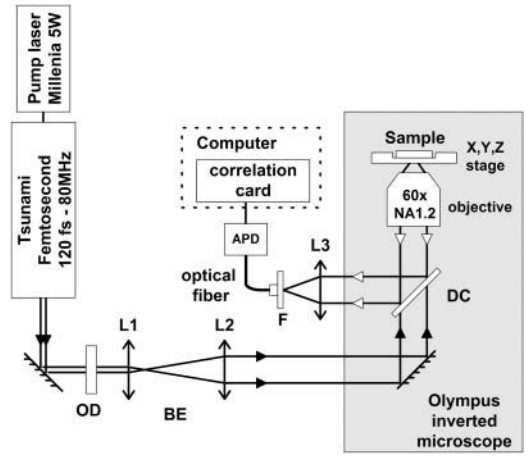

FIGURE 1 Diagram of the experimental two-photon excitation FCS setup. (*OD*) optical density filter; (*BE*) beam expander made of two convergent lenses (L1/L2); (*DC*) dichroic beamsplitter, (*F*) BG39 filter; (*APD*) avalanche photodiode.

Calibration of the system was performed with a 50-nM TMR solution. Assuming a diffusion constant of 2.8×10^{-10} m^2 s^{-1} (Rigler et al., 1993), the equatorial (r_0) and axial (z_0) radii of the focal volume are respectively of 0.29 and 1.3 μm, giving an effective volume ($V = (\pi/2)^{3/2} r_0^2 z_0$) (Schwille et al., 1999) of 0.2 fl.

FCS data analysis

Assuming a three-dimensional Gaussian distributed excitation intensity, the fluorescence ACF of free diffusing species can be calculated by:

$$G_{\mathrm{D}}(\tau) = \frac{1}{N}\left(\frac{1}{1+(\tau/\tau_{\mathrm{D}})}\right)\left(\frac{1}{1+(S)^2(\tau/\tau_{\mathrm{D}})}\right)^{1/2} \times \left(1+\frac{Fe^{-\tau/\tau_{\mathrm{t}}}}{1-F}\right), \quad (1)$$

where N is the mean number of molecules in the excitation volume, S is the ratio between the equatorial and axial radii of the focal volume, and τ_{D} is defined as the characteristic diffusion time of the particle. τ_{t} designates the triplet state lifetime and F is the mean fraction of fluorophores in the triplet state.

In the case of a multicomponent system with species of different quantum yields, a more general form of Eq. 1 may be used (Thompson, 1991):

$$G(\tau) = \sum_{\mathrm{i}=1}^{\mathrm{M}} q_{\mathrm{i}}^2 N_{\mathrm{i}}^2 G_{\mathrm{Di}}(\tau) \Big/ \left[\sum_{\mathrm{i}=1}^{\mathrm{M}} q_{\mathrm{i}} N_{\mathrm{i}}\right]^2, \quad (2)$$

where N_{i} and $G_{\mathrm{Di}}(\tau)$ are the mean number and the ACF due to diffusion of the *i*th species in the focal volume. q_{i} designates the ratio of the fluorescence yield (given by the product of the detection efficiency by the absorption cross section and the fluorescence quantum yield) of the *i*th species to that of species 1, taken as a reference.

FCS data were analyzed either with Origin (Microcal, Northhampton, MA) for discrete diffusion times or with a software based on the maximum

entropy method (Livesey and Brochon, 1987; Swaminathan and Periasamy, 1996) for distribution of diffusion times. This last software was supplied by N. Periasamy (Mumbai, India). The weight function for the FCS data is scaled suitably to produce a uniform distribution of weighted residuals for a good fit using standards.

RESULTS

FCS measurements on free DNA and PEI

As a first step, TPE FCS was performed on free rhodamine-labeled DNA plasmid (5.1 kbp). The experimental $G(\tau)$ function was satisfactorily fitted to Eq. 1 (Fig. 2). The triplet lifetime and the fraction of molecules in the triplet state were respectively 25 ($\pm$10) μs and 0.24 ($\pm$0.02). Using TMR as a reference, the diffusion coefficient, D_{DNA}, of the plasmid was calculated by: $D_{DNA} = D_{TMR} \times \tau_{D(TMR)}/\tau_{D(DNA)}$. This leads to $D_{DNA} = 3.9\ (\pm 0.7) \times 10^{-12}\ m^2\ s^{-1}$, in reasonable agreement with the value previously reported for a slightly shorter plasmid (Kral et al., 2002b). The D_{DNA} value may be further compared with the theoretical one, D_{th}, calculated for a circular wormlike chain model (Bloomfield et al., 2000) by:

$$D_{th} = \frac{kT}{6\pi\eta(0.665\langle R_g^2\rangle^{0.5})}, \quad (3)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the buffer. The radius of gyration, $\langle R_g^2\rangle$, is given by: $\langle R_g^2\rangle = (1/12)b^2N$ where b and N are the length and the number of statistical segments in the plasmid DNA. The b parameter is given by two times the persistence length, which is ~500 Å for B-DNA (Bloomfield et al., 2000). Assuming a rise of 3.4 Å/bp, the contour length of the plasmid DNA is ~17,340 Å and thus N is ~17. This leads to $D_{th} = 2.7 \times 10^{-12}\ m^2\ s^{-1}$. In respect with the various assumptions used to calculate D_{th} its value is in reasonable agreement with the experimental value.

In a next step, we investigated by FCS, the behavior of free TRITC-labeled PEI in solution. Surprisingly, despite PEI polydispersity, the autocorrelation data (Fig. 3 *A*) were adequately fitted by Eq. 1, which assumes a discrete correlation time. Moreover, attempts to treat the data with software based on the maximum entropy method that allows to recover distributions of correlation times do not significantly improve the fit (data not shown). These observations are probably linked to the rather limited dependence of the diffusion coefficients (and thus the correlation time) on the molecular weight (Meseth et al.,

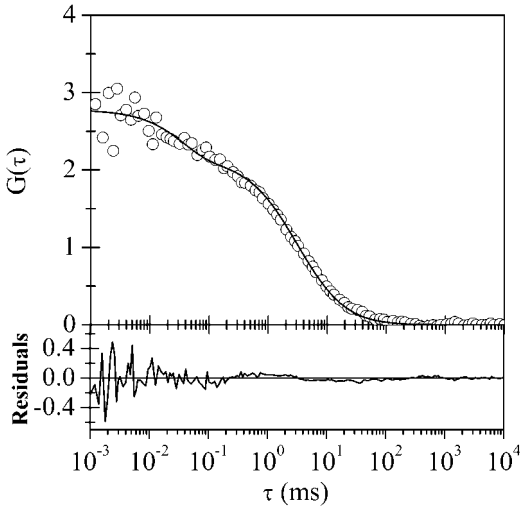

FIGURE 2 Autocorrelation curve of rhodamine-labeled plasmid. The solid line corresponds to the fit of the experimental curve (*circles*) by Eq. 1 with the parameters given in the text. The concentration of DNA was 20 μM. Residuals of the fit are shown in the lower panel. A slight improvement of the fit was observed by using a two-component equation but the additional component was found to be poorly reproducible and was thus disregarded.

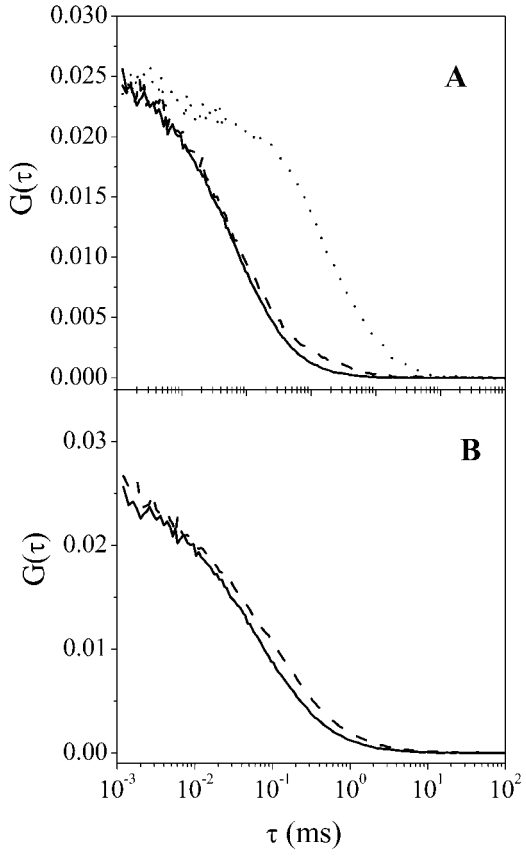

FIGURE 3 Autocorrelation curves of free PEI, and PEI/DNA and PEI/heparin complexes. (*A*) The autocorrelation curves of free PEI (*solid line*) and PEI/DNA complexes (*dashed line*) were performed with the same concentration of labeled PEI (200 μM). The N/P ratio used for the formation of the complexes is 10. The dotted line corresponds to the autocorrelation curve of PEI/DNA complexes obtained in the same conditions but with rhodamine-labeled DNA. For comparison, this curve was normalized to the curves obtained with labeled PEI. (*B*) The autocorrelation curves of free PEI (*solid line*) and PEI/heparin (*dotted line*) were obtained with a 200 μM concentration of labeled PEI. The concentration of heparin was 1mg/ml. The autocorrelation curve obtained after addition of heparin to PEI/DNA complexes was indistinguishable from the PEI/heparin curve and is thus not represented. For the sake of clarity, only the experimental curves (and not the fits) are represented.

1999). It results from this dependence that for example, a change of the molecular weight by a factor of eight changes only the correlation times by a factor of two. In addition, it is likely that as reported for an 11.9 kDa PEI (Fischer et al., 1999), the polydispersity of our PEI batch may be rather small. In keeping with this last conclusion, our data further suggest that the probable heterogeneity in the number of fluorophores per PEI molecule resulting from PEI polydispersity is limited and does not strongly affect the analysis of the autocorrelation curves.

From the correlation times of PEI, a value of 1.2 ($\pm$0.2) $\times$ 10^{-10} m^2 s^{-1} was calculated for the mean diffusion constant, D_{PEI}, of PEI. Assuming that PEI molecules are nearly spherical (Hellweg et al., 2000), the mean hydrodynamic radius, R_h, of PEI can be deduced from the Stokes-Einstein relation: $R_h = kT/6\pi\eta D_{PEI}$. The 1.8-nm value deduced for R_h was significantly smaller than the 4.7-nm and 6.6-nm radius previously determined by dynamic light scattering (Andersson et al., 2000; Hellweg et al., 2000). Because in polydisperse samples, the apparent diffusion coefficient is a weighted average of the contributions from the various molecules in solution, differences in the weighting factors attributed by dynamic light scattering and FCS to the various species may partly explain the different R_h values. It should be noted that a population with a slower diffusion constant (3.8 ($\pm$0.3) $\times$ 10^{-11} m^2 s^{-1}) is detected in some autocorrelation curves. Nevertheless, its small relative contribution clearly suggests that it corresponds to a minor species. Moreover, the diffusion constant of these minor species is significantly different from that of PEI/DNA complexes and may thus, not interfere with their measurements.

The second information extracted from the FCS curves is the mean number, N, of molecules present in the focal volume. This number corresponding to the inverse of $G(0)$ was measured for the three concentrations (80, 120, and 200 μM) of PEI that were used to prepare PEI/DNA complexes with an N/P ratio of 4, 6, and 10, respectively. The measured numbers (14, 21, and 39 respectively) were in good agreement with the theoretical ones, N_{th}, (16, 24, and 41, respectively) calculated by:

$$N_{th} = \frac{C_{PEI} \times N_A}{P^\circ} \times V, \quad (4)$$

where P° (=580) is the polymerization degree of PEI, V is the focal volume, C_{PEI} is the molar concentration of PEI expressed as the total nitrogen concentration, and N_A is Avogadro's number. Noticeably, a time-dependent increase of the FCS count rate, correlated with an increase of N, and followed by the reaching of a plateau after 20 min was observed (data not shown). Because the 300-μl volume of the measurement cell was sufficient to prevent significant evaporation and because a similar time-dependent change of the steady-state fluorescence intensity was observed in conventional quartz cells, this variation may be attributed to an adsorption-desorption process of PEI onto the glass or quartz surface. As a consequence, to allow stabilization of the system, the solutions were left for at least 20 min in the wells before FCS acquisitions.

FCS measurements on PEI/DNA complexes

To investigate the diffusion behavior of PEI/DNA complexes, two different approaches were used. First, complexes were prepared using the rhodamine-labeled plasmid and nonlabeled PEI at N/P ratios of 4, 6, and 10, respectively. The fluorescence fluctuation profiles at the three N/P ratios are shown in Fig. 4. It could be readily observed that at the lowest ratio, the profile is dominated by a few but highly intense (with an intensity 50 times larger than the fluorescence background) peaks. These peaks may be unambiguously attributed to the large multimolecular complexes that form at this ratio (J.S. Remy, personal communication). Moreover, in excellent agreement with the reported decrease in PEI/DNA complex size with increasing N/P ratios (Erbacher et al., 1999), an increase in the number of peaks as

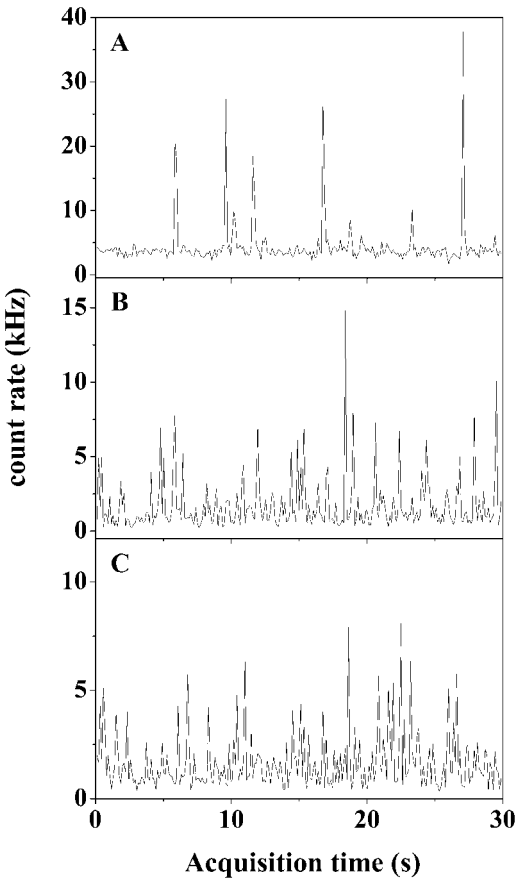

FIGURE 4 Fluorescence fluctuations of PEI/DNA complexes in the focal volume. Complexes of rhodamine-labeled plasmid with PEI were formed at N/P ratios of 4 (*A*), 6 (*B*), and 10 (*C*), respectively with a DNA concentration of 20 μM.

well as a decrease in their intensity were observed for the higher ratios.

In keeping with previous reports on complexes of DNA with synthetic vectors (Van Rompaey et al., 2001), the few intense peaks at N/P = 4 prevented an analysis of the fluctuation profile by the ACF. In contrast, autocorrelation curves were readily obtained and adequately fitted by Eq. 1 at N/P ratios of 6 and 10 (Fig. 5). A mean diffusion coefficient of 6 ($\pm$1) $\times$ 10^{-12} m^2 s^{-1} was obtained for both ratios. Because the PEI/DNA complexes show rounded, globular forms (Dunlap et al., 1997), their hydrodynamic diameter may be rigorously deduced from the Stokes-Einstein equation. Particle sizes of 70 ($\pm$10) nm were calculated, in good agreement with the 57 ($\pm$14) nm sizes determined by light scattering measurements (Blessing et al., 2001). At both N/P ratios of 6 and 10, the formation of the complexes increases the $G(0)$ value and thus decreases the number of species by a factor of 3.5 ($\pm$1) as compared to free DNA. If we reasonably assume that all DNA molecules were complexed at both N/P ratios, it follows that each complex may contain an average of 3.5 ($\pm$1) plasmids.

In a second approach, the complexes were formed by using TRITC-labeled PEI and nonlabeled plasmid. The autocorrelation curves of the complexes formed at N/P ratios of 6 and 10 were similar to the curves obtained with the corresponding concentrations of free PEI (Fig. 3 *A*). In fact, the only difference is the small shoulder for long times in the autocorrelation curve of the complexes. This suggests that a large proportion of PEI remains free when PEI is mixed with DNA and/or that the fluorescence of the complexes is rather low. The autocorrelation curves could be adequately fitted with Eq. 2 if we assume the existence of two species. If the correlation times of the two species were allowed to float, we obtain values typical of free PEI and complexes, respectively. Of course, according to its small contribution, the correlation time of the complex fluctuates in a larger range than that of free PEI. Therefore, to achieve reasonable fitted amplitudes for the two species, the values of the correlation times were held fixed in the subsequent steps (Meseth et al., 1999). In this respect, the populations of free and bound species were determined from their respective amplitudes A_1 and A_2 (obtained from the fits of the autocorrelation data to Eq. 2) by using the following system:

$$\begin{cases} A_1 = \dfrac{N_1}{(N_1 + qN_2)^2} \\ A_2 = \dfrac{q^2 N_2}{(N_1 + qN_2)^2} \end{cases}, \tag{5}$$

where q is the ratio of the fluorescence yield of the complexes to that of free PEI. The number of complexes, N_2, was determined independently from the FCS curves of complexes formed with rhodamine-labeled plasmid and unlabeled PEI in the same conditions. Resolution of the system (Eq. 5) provides the N_1 and q values reported in Table 1. For both N/P ratios, the comparison with the autocorrelation curves of the corresponding PEI concentrations without DNA shows that only ~15% of the PEI molecules are complexed to DNA. By comparing the numbers of bound PEI molecules and complexes, we further deduced that the mean number of PEI molecules per complex was ~30. From this number, it could be expected that if no fluorescence change accompanies the binding process, the complexes would be 30 times brighter than free PEI molecules. In contrast to this expectation, the fluorescence ratio between the complexes and free PEI (as measured by q) was found to be as low as 3.2 ($\pm$0.2), suggesting that the labeled PEI molecules are strongly quenched in the complexes. This is likely due to the close proximity of the dyes that may interact through dipole-dipole coupling as described by the exciton theory (Bernacchi and Mely, 2001; Packard et al., 1996; Wang et al., 2000). Because in addition to a strong decrease in fluorescence

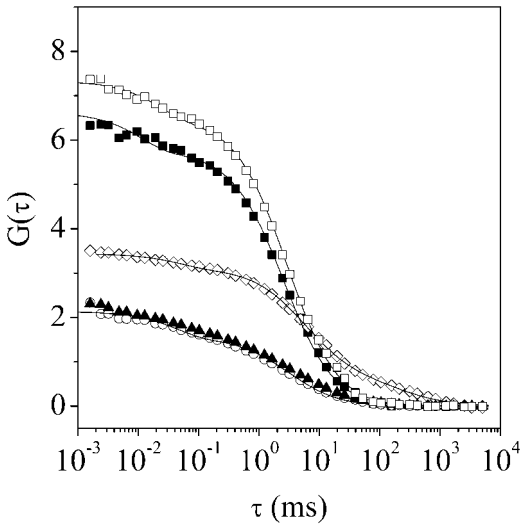

FIGURE 5 Formation and heparin-induced dissociation of complexes of PEI with rhodamine labeled plasmid. The experimental autocorrelation curves of free plasmid DNA (*open circles*) and PEI/DNA complexes (*open squares*) were fitted (*solid lines*) with Eq. 1. Three different concentrations (0.03 (*closed squares*), 0.3 (*diamonds*), and 1 (*closed triangles*) mg/ml) of heparin were added to PEI/DNA complexes. In each case, the DNA concentration was 20 μM and the N/P ratio was 10.

TABLE 1 Determination of the amounts of free and bound PEI in a mixture with DNA

N/P	N*	N_1*	N_2*	N_b*	q*	Free PEI* (%)
6	21 ± 2	18 ± 2	0.12 ± 0.04	3	3.7 ± 0.7	86 ± 1
10	39 ± 4	34 ± 4	0.15 ± 0.02	5	3.2 ± 0.2	87 ± 4

*N is the number of PEI molecules in a DNA-free solution, determined independently with a PEI solution at the same concentration as the mixture. N_1 and N_2 are the number of free PEI molecules and complexes, respectively. The number, N_b, of bound PEI molecules is given by: $N_b = N - N_1$. q is the ratio of the fluorescence yield of the complexes to that of free PEI.

intensity, this kind of interaction is expected to modify the absorption properties of the dyes, we compare the absorption spectrum of free labeled PEI with the spectrum obtained by mixing labeled PEI with DNA. Despite the large excess of free PEI in the mixture, a significant red shift of the 550-nm shoulder could be observed (Fig. 6). This red shift was significantly increased when the relative concentration of complexes was increased by filtration. To confirm the interaction between the labeled PEI molecules, FCS measurements were performed with complexes formed by adding DNA to a mixture of labeled and unlabeled PEI (in a 1:4 ratio). Although the number of labeled molecules was divided by four, the q ratio decreases only by a factor of two. This suggests that the decrease of the number of labeled PEI reduces the number of interchromophore interactions and thus, the quenching. Moreover, because the fraction of free PEI was similar to that measured when only labeled PEI was used, this suggests that labeled and unlabeled PEI interact similarly with DNA (data not shown).

Monitoring of PEI/DNA complex purification by ultrafiltration

As a first application of the determination by FCS of the concentrations of free and bound partners in the interaction of DNA with PEI, we analyzed the purification of the complexes by an ultrafiltration procedure previously described (Finsinger et al., 2000). The complexes were prepared at an N/P ratio of 10 and then submitted to several filtration steps as described in Materials and Methods. The mixtures of DNA with PEI were analyzed by FCS before and after filtration. If the diffusion time of the complexes was allowed to float, it appeared that the filtration slightly but reproducibly increases the diffusion constant of the complexes (Table 2). This may be attributed to a preferential retention of the bigger complexes onto the filter.

To assess the ultrafiltration efficiency, the ratio, R, between the number of free PEI molecules (N_1) and the number of complexes (N_2) measured in the focal volume, could be used as a relevant criteria (Table 2). This ratio, which also corresponds to the concentration ratio, was ~280 before filtration and decreased to ~90 after filtration. This suggests that the complexes were purified by a factor of 3.2 and thus the contribution of free PEI decreased to ~60%. Moreover, from the knowledge of the volumes of solution before and after filtration, it was further possible to deduce that 35% of the complexes were lost on the filter during the filtration.

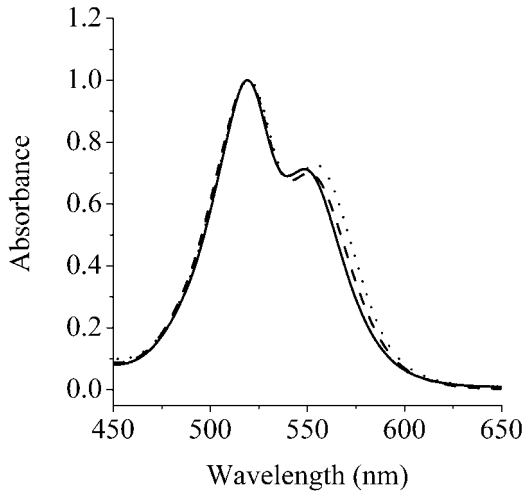

FIGURE 6 Absorption spectra of free PEI and PEI/DNA complexes. The solid line corresponds to the absorption spectrum of free TRITC-labeled PEI. The dashed and dotted lines correspond to the absorption spectra of PEI/DNA complexes before and after filtration, respectively. The spectra were normalized to the maximum absorbance peak of free PEI. The concentrations of PEI and DNA were as described in Fig. 3.

Monitoring of heparin-induced dissociation of PEI/DNA complexes

Heparin has been recently reported to release DNA from its complexes in a concentration-dependent manner (Moret et al., 2001). To check this point and quantify this release, FCS was used to monitor the dissociation of the complexes at various heparin concentrations (0.01, 0.03, 0.1, 0.3, 1, 3 mg/ml). Using complexes with rhodamine-labeled DNA, the similarity of the diffusion constants of free DNA and complexes prevented the observation of the two populations. In contrast, the heparin-induced release of DNA could be evidenced from the large differences between the $G(0)$ values of free DNA and PEI/DNA complexes (Fig. 5). Because the autocorrelation curves at heparin concentrations $\geq$1 mg/ml were indistinguishable from the autocorrelation curve of free DNA, this suggests that at these concentrations, heparin induces a total dissociation of the PEI/DNA complexes. In contrast, no dissociation could be observed for heparin concentrations $\leq$0.03 mg/ml. At intermediate heparin concentrations, the autocorrelation curves indicate at least two populations of large particles with diffusion coefficients of 1.8×10^{-12} m^2 s^{-1} and 4×10^{-14} m^2 s^{-1}. This suggests the formation of large heparin/PEI/DNA aggregates.

TABLE 2 Ultrafiltration of PEI/DNA complexes

	D_1* ($\times 10^{-10}$) (m^2 s^{-1})	D_2* ($\times 10^{-12}$) (m^2 s^{-1})	N_1/N_2*	Free PEI† (%)
Before filtration	1.3 ± 0.2	6 ± 1	280 ± 40	83 ± 5
After filtration	1.1 ± 0.1	8 ± 2	90 ± 10	59 ± 2

*D_1 and D_2 are the respective diffusion constants of free and bound PEI, respectively. N_1 and N_2 are the number of free PEI molecules and complexes, respectively. Complexes were made with 100 μM of DNA and an N/P ratio of 10.

†The percentage of free PEI was calculated from N_1 and N_2 by assuming that each complex is composed of 3.5 plasmids and 30 PEI molecules.

To further confirm the heparin-induced dissociation of PEI/DNA complexes, a final heparin concentration of 1 mg/ml was tested on complexes with labeled PEI. The obtained autocorrelation curve could be adequately fitted with a diffusion constant half that of free PEI and was indistinguishable from the autocorrelation curve obtained by adding heparin directly to free PEI (Fig. 3 *B*). This clearly suggests that the newly formed species may correspond to complexes of PEI with heparin.

DISCUSSION

The aim of the present study was to quantify by FCS with TPE the amounts of free and bound partners in mixtures of plasmid DNA with PEI. To achieve this goal, experiments were performed with rhodamine-labeled PEI or plasmid DNA. By mixing PEI with DNA at N/P ratios of 6 or 10 that were reported to give optimal transfection efficiencies (Finsinger et al., 2000), we found that ~86% of PEI was in a free form and that each complex contains an average of 3.5 plasmids and thirty molecules of PEI. The number of plasmids in PEI/DNA complexes is significantly smaller than the number (15) of 5.1 kbp plasmids that would be contained in the spherical complexes (of the same size as the PEI/DNA complexes) formed by interaction with dimerizable detergents (Blessing et al., 1998). A similar conclusion can be drawn from the comparison with the toroidal condensates obtained from the interaction of DNA with multivalent cations because it has been calculated that toroids with a volume slightly lower than that of PEI/DNA complexes contain ~13 plasmids of 3 kbp (Bloomfield, 1991). Both comparisons suggest that PEI compacts the plasmid DNA less tightly than the smaller condensing agents. This may be a consequence of the bulky scaffold formed by the branched PEI molecules that sterically prevents a strong compaction of the DNA molecules. To further strengthen our hypothesis, we calculate the fractional volume occupancy of DNA in the complex. For this purpose, the volume of dry DNA is calculated by $V_{DNA} = \pi a^2 L$, where a and L are the radius and length of the DNA, taken as a rod (Bloomfield, 1991). Using $a = 10$ Å and $L = 5100 \times 3.4 = 17{,}300$ Å, we calculate a total volume of 1.9×10^{-23} m^3 for 3.5 plasmids. The comparison with the 1.8×10^{-22} m^3 volume of a spherical PEI/DNA complex with a diameter of 70 nm revealed that ~10% of the PEI/DNA complex volume is occupied by DNA. This fraction is significantly less than the 72% value reported for condensates of DNA with multivalent cations (Bloomfield, 1991) or the 75% value calculated from the data of DNA condensed by dimerizable detergents (Blessing et al., 1998).

Moreover, because a pK_a of 8.4 has been recently reported for the 25 kDa PEI used in our study (von Harpe et al., 2000), we may additionally calculate the number of positive charges in the complex. From the mean molecular weight of PEI, it may be calculated that each PEI contains ~580 nitrogen atoms. Moreover, according to the pK_a of PEI, it results that at pH = 7.4, ~90% of the nitrogens are protonated. It follows that the number of positive charges in the complex is ~16,000 and is thus significantly lower than the number of phosphate groups (36,000) in the complex. Moreover, it may be noted that even lower pK_a values have been reported earlier for PEI (Suh et al., 1994), and may thus lead to complexes with an even lower +/− charge ratio. These findings are apparently in contrast with the well-documented need for a condensing agent to neutralize ~90% of DNA charges to induce its condensation (Wilson and Bloomfield, 1979). However, the latter rule has been established with small or linear multivalent cations and may not necessarily apply to the highly branched PEI molecules. We may thus speculate that according to the large size and the reticulation of PEI as well as to the structural constraints of DNA, the wrapping of DNA around the PEI molecules may not necessarily neutralize all the DNA charges but may cause the localized bending or distortion of the DNA required to facilitate condensation (Bloomfield et al., 2000). The lack of DNA charge neutralization may be especially true for the interior of the complex, where steric constraints may prevent the binding of the additional PEI molecules that would be required to provide electroneutrality. Moreover, the only partial neutralization of the DNA charges may prevent a close packing of the DNA molecules and further contribute to the limitation of the number of DNA molecules in the complex. In contrast to the complex interior, additional PEI molecules may bind at the surface and thus, explain the strong positive surface charge of the complexes (Godbey et al., 1999a).

The most striking feature of the composition of mixtures of DNA with PEI is the large amount of PEI that remains in free form. This is a major concern for transfection protocols because PEI has been reported to induce cell dysfunction (Godbey et al., 2001) and be cytotoxic (Fischer et al., 1999; Godbey and Mikos, 2001; Godbey et al., 2001; Putnam and Langer, 1999). This cytotoxicity seems to be related in part to the high affinity binding of PEI as huge clusters on the outer surface of the plasma membrane (Fischer et al., 1999). The deposition of these clusters may impair the plasma membrane functions and lead to cell death by necrosis. In addition, free PEI has been shown to destabilize the outer membranes of Gram-negative bacteria (Helander et al., 1997, 1998) as well as liposomal membranes made from phosphatidyl serine (Oku et al., 1986). Moreover, PEI at high concentration has also been reported to cause lysosomal disruption in rat hepatocytes (Klemm et al., 1998). However, the role of PEI in membrane permeabilization is still debated (Godbey et al., 1999b). An additional source of induction of cellular dysfunction and toxicity may arise from the ability of PEI to enter into the cell nucleus (Godbey et al., 1999c) where it may interact with the host DNA and alter host transcriptional processes (Godbey et al., 2001). Interaction of PEI with the nucleus may induce a delayed cell death (7–9 h posttransfection) by opposition to the more immediate (2 h

posttransfection) cell death associated with the interaction of PEI with the membranes (Godbey et al., 2001).

Both types of cell death would probably benefit from a decrease in the level of free PEI in the mixture of PEI and DNA used for transfection. Among the methods proposed to reach this aim, we have tested the efficiency of the ultrafiltration method proposed by Finsinger et al. (2000). We found that this method removes ~30% of the free PEI. This percentage is slightly less than that previously reported (Finsinger et al., 2000), a difference that may tentatively be ascribed to the higher proportion of complexes lost by adsorption onto the filter at our lower DNA concentration. Indeed, because adsorption depends on the mass rather than the concentration of adsorbing species (Cantor and Schimmel, 1980), its relative contribution increases at lower concentration.

The only partial purification of the PEI/DNA complexes by ultrafiltration may be attributed to their limited stability. Such a limited stability has already been reported for complexes of DNA with low molecular weight PEI as a consequence of the insufficient number of salt bonds to provide a tight complex (Godbey et al., 1999a). The most labile PEI molecules are likely those bound at the surface of the complexes. In agreement with this hypothesis, a significant decrease of the surface charge of the PEI/DNA complexes was shown to follow the decrease of free PEI concentration by ultrafiltration (Finsinger et al., 2000) or centrifugation (Godbey et al., 1999a). The removal of surface-bound PEI molecules would also be consistent with the increased diffusion constant (and thus, the reduced diameter) of the complexes after ultrafiltration (Table 2).

TPE FCS was also used to monitor the heparin-induced dissociation of PEI/DNA complexes. Noticeably, a 10-fold lower heparin concentration than that previously reported (Moret et al., 2001) was required to dissociate PEI/DNA complexes. This may probably be ascribed to the increased affinity of heparin for PEI/DNA complexes in our low salt conditions. Moreover, our results suggest that at heparin concentration between 0.03 and 1 mg/ml, heparin forms large aggregates with PEI/DNA complexes. We speculate that at these concentrations, heparin binds to the external PEI molecules of the PEI/DNA complexes and cross-bridges various complexes together.

In conclusion, TPE FCS appears as an adequate tool to determine the concentrations of free and bound partners in mixtures of DNA and PEI. This technique was shown to be useful for monitoring the removal of free DNA by filtration techniques as well as the dissociation of PEI/DNA complexes by heparin. Because TPE FCS is ideally suited for measurements in the cells (Hess et al., 2002; Schwille, 2001; Schwille et al., 1999), this technique may be used to monitor the dissociation of PEI/DNA complexes during their intracellular pathway. However, because large amounts of free PEI hinder an accurate determination of the number of complexes, efforts should be done to transfect cells with mixtures of DNA with PEI containing a reduced amount of PEI. These experiments are currently in progress.

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