Supporting information

A study of complexation and biological fate of Polyethyleneimine-siRNA polyplexes in vitro and in vivo by means of Fluorescence Correlation Spectroscopy and Positron Emission Tomography Imaging

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Table S1: Values of diffusion coefficient (D) and hydrodynamic diameter (D_H) at different N/P ratio obtained from the in vitro measurements.

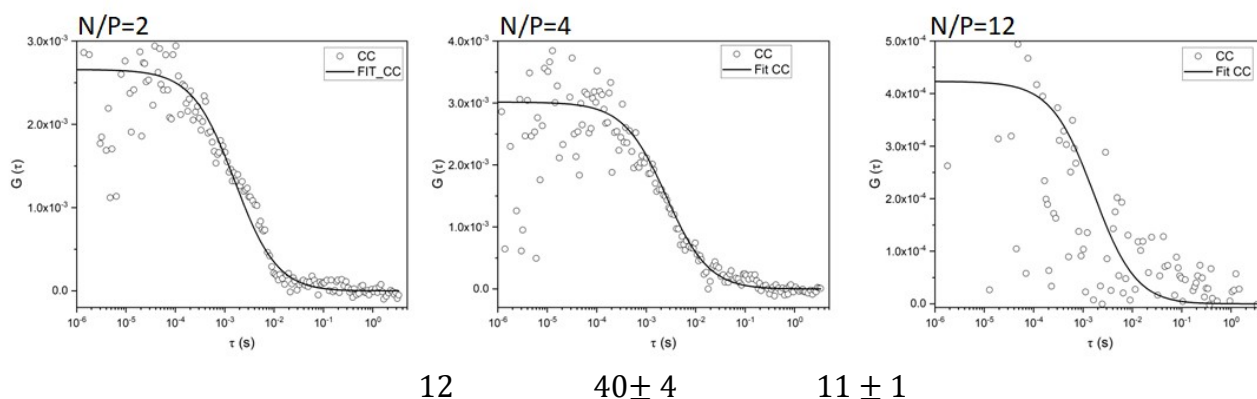| N/P_{Theo}	$D\ (\mu m^2/s)$	$D_H (nm)$
2	18 ± 6	27 ± 8
4	16 ± 6	34 ± 16
12	40 ± 4	11 ± 1

Figure S1: The resulting cross-correlation functions (CC) for the different N/P ratios from in vitro experiments.
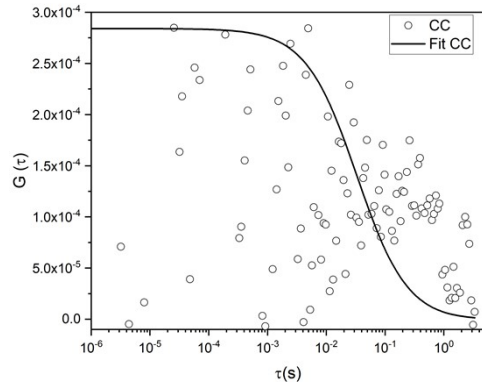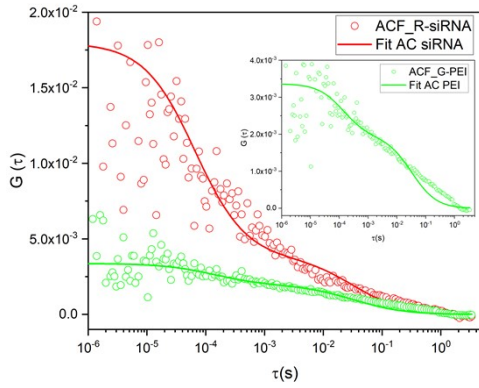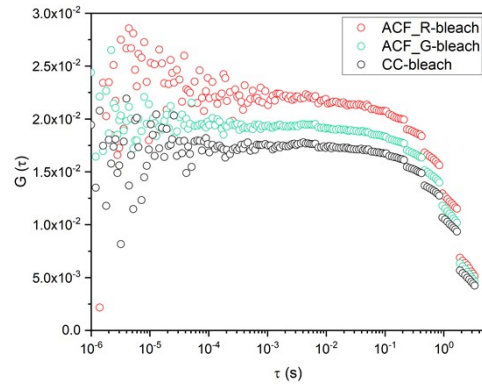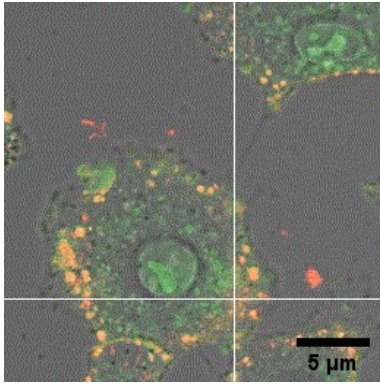
N/P=2, cells, day 1

Figure S2: Measurement of the cross-correlation of PEI and siRNA directly after transfection of cells (top left corner). The cross hair shows the position of measurement. Due to immobile PEI and siRNA a bleaching was performed before FCS measurements, leading to a decrease of the signal intensity (top right corner). The resulting autocorrelation functions, red and green curves, and the cross-correlation function in black are shown at the bottom (left and right, respectively). After 24 h the CC decreased in comparison to the measurement directly after the transfection.
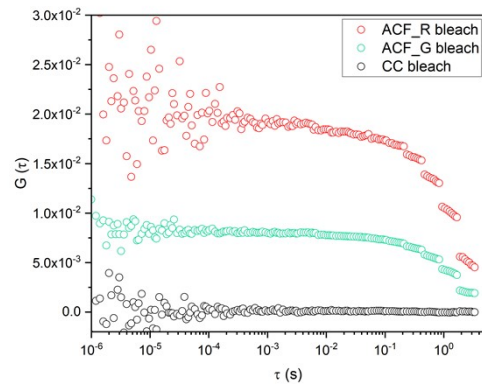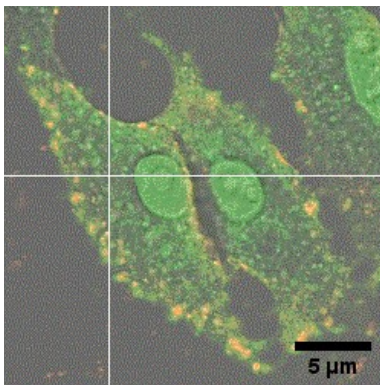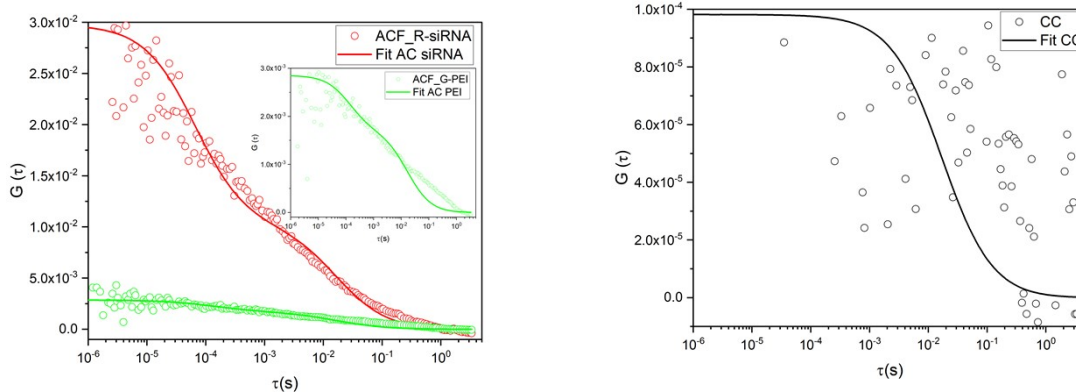
N/P=2, cells, day 2

Figure S3: Measurement of the cross-correlation of PEI and siRNA directly after transfection of cells (top left corner). The cross hair shows the position of measurement. Due to immobile PEI and siRNA a bleaching was performed before FCS measurements, leading to a decrease of the signal intensity (top right corner). The resulting autocorrelation functions, red and green curves, and the cross-correlation function in black are shown at the bottom (left and right, respectively). After 48 h the CC decreased in comparison to the measurement directly after the transfection.

Table S2: diffusion coefficient (D), hydrodynamic diameter (D_H) and the cross-correlation amplitude A_{CC} at N/P ratio 2 and 4 obtained from the intracellular measurements 1 and 2 days after the transfection.

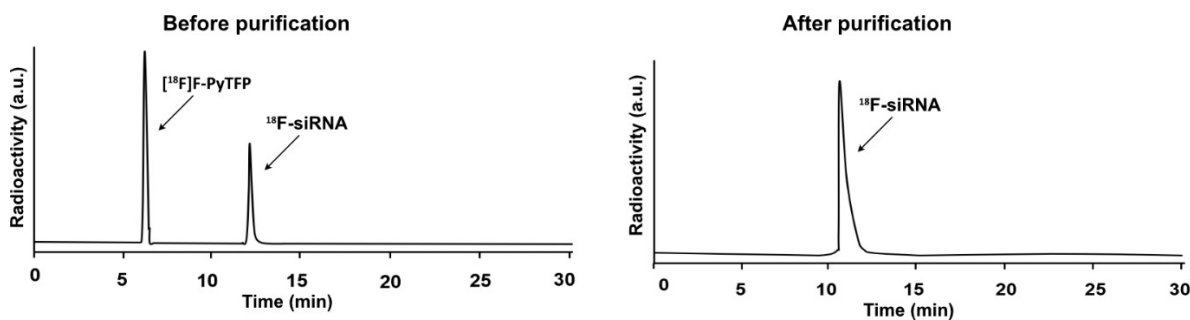| N/P_{Theo}	$D\ (\mu m^2/s)$	$D_H(nm)$	$A_{CC} \cdot 10^{-5}$
2, day 1	0.27 ± 0.4	1643 ± 364	20 ± 7
2, day 2	0.5 ± 0.4	1274 ± 787	14 ± 5
4, day 1	0.3 ± 0.5	1701 ± 859	42 ± 7
4, day 2	1.1 ± 0.6	627 ± 429	4 ± 2

Figure S4. Representation of the chromatograms obtained before and after purification of ^{18}F-siRNA performed by high performance liquid chromatography with radioactivity detector (radio-HPLC).
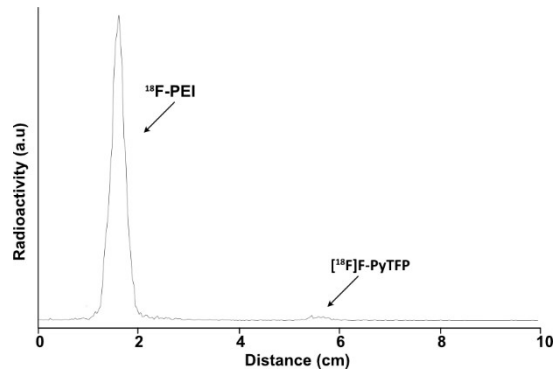
Figure S5. Radio-thin layer chromatography (radio-TLC) obtained during the radiolabelling of PEI.
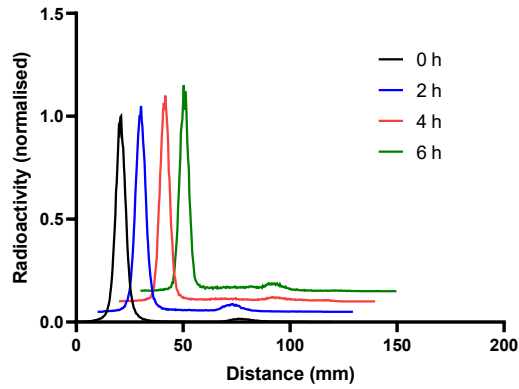
Figure S6. Radio-thin layer chromatography (radio-TLC) obtained for stability studies of [^{18}F]PEI at 2, 4 and 6 h after preparation. The peak at low Rf corresponds to [18F]PEI, while the other peak corresponds to unbound prosthetic group.
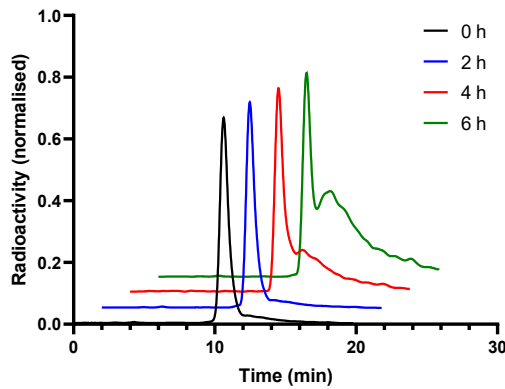
Figure S7. Radio-high performance liquid chromatography (radio-HPLC) profiles obtained for stability studies of [^{18}F]SiRNA at 2, 4 and 6 h after preparation. The peak with retention time of ca. 11 min corresponds to [^{18}F]SiRNA, while the other peak, appearing at t>4 h corresponds to an unidentified radioactive specie.

Figure S8. Concentration of radioactivity in brain, stomach and spleen at different time points after intravenous administration of PEI/^{18}F-siRNA and ^{18}F-PEI/siRNA polyplexes, determined by PET imaging. Results are expressed as % of injected dose per cm^3 of tissue (%ID/cm^3; average ± standard deviation, n = 3).
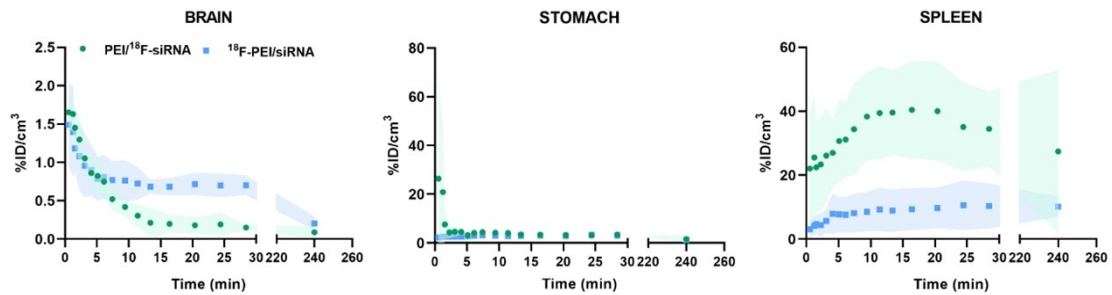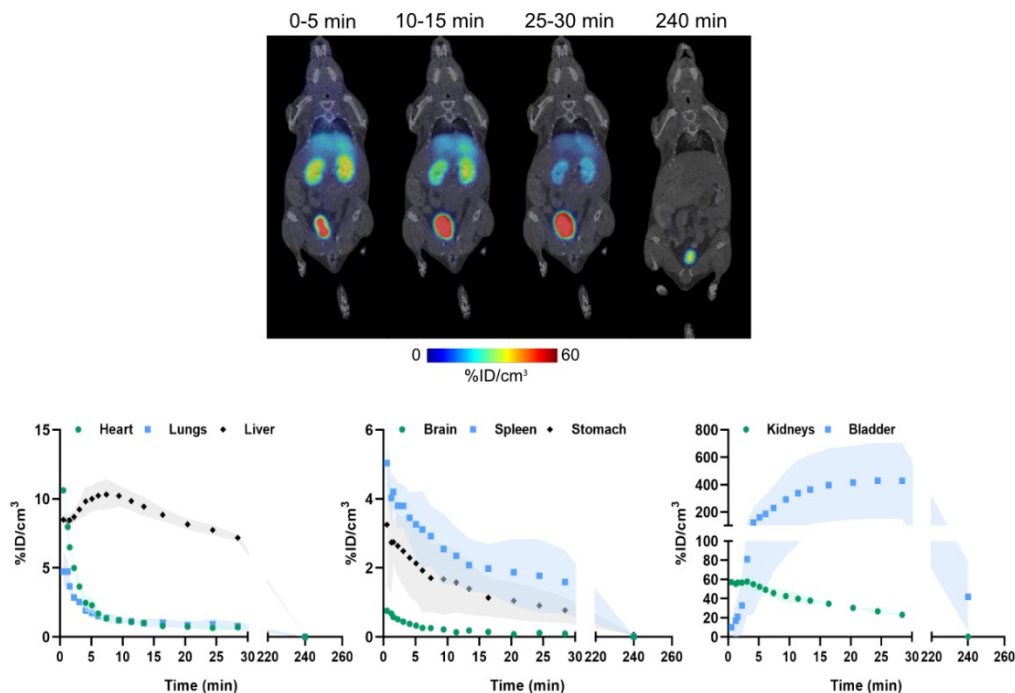

Figure S9. Top, representative PET images (coronal views, maximum intensity projection) obtained after intravenous administration of ^{18}F-siRNA at different time points. Images were co-registered with representative CT slices. Bottom, concentration of radioactivity in different organs after administration of ^{18}F-siRNA at different time points. Results are expressed as % of injected dose per cm^3 of tissue (%ID/cm^3; average ± standard deviation, n = 3).

Calculation of the effective N/P ratio from autocorrelation functions

From the in vitro FCCS measurements for all N/P ratios we observed an excess of PEI. Based on this, we can assume that siRNA is entirely complexed by PEI. Hence, the number of diffusing species, which are described by the autocorrelation function of the siRNA (ACF_R-siRNA), is equal to the number of polyplexes (N_{PP}) plus the number of free dye (N_{Cy5}):

$$N_{PP} + N_{Cy5} \propto \frac{1}{G(0)_{siRNA}}$$

Eq. S1

In order to estimate the effective N/P ratio, first we have to calculate the fractions of the different species: free dyes, PEI and siRNA (free and forming the polyplexes). To do this, is necessary to obtain the diffusion times of them by means of their autocorrelation functions. The fitting procedure is summarized in the next scheme S1.

siRNA analysis

Step	Data file	Fitting model	Result
1	Calibration of the confocal volume with ATTO 633	3D normal diffusion 1-component fit	γ, ω_{xy}
2	Fitting of the ACF of siRNA alone	3D normal diffusion 2-component fit γ, ω_{xy} and D_{cy5} fixed	τ_2
3	Fitting of the ACF_R-siRNA	3D normal diffusion 3-component fit γ, ω_{xy}, D_{cy5} and τ_2 fixed	τ_3, N_{PP}, ρ_1, ρ_2, ρ_3

PEI analysis

Step	Data file	Fitting model	Result
1	Calibration of the confocal volume with Rhodamine 123	3D normal diffusion 1-component fit	γ', ω'_{xy}
2	Fitting of the ACF of the Green Rhodamine	3D normal diffusion 1-component fit γ' and ω'_{xy} fixed	τ'_1
3	Fitting of the ACF of PEI alone	3D normal diffusion 2-component fit γ', ω'_{xy} and τ'_1 fixed	τ'_2
4	Fitting of the ACF_G-PEI	3D normal diffusion 3-component fit γ', ω'_{xy}, τ'_1, τ'_2 and τ'_3 fixed	ρ'_1, ρ'_2, ρ'_3

Scheme S1: Schematic overview of the fitting steps in order to estimate the number of siRNA and PEI molecules associated in the polyplex, as well as the effective N/P ratio. This requires a prior 1-component fitting of the corresponding fluorophore to calibrate the effective volume of each wavelength (Rhodamine 123 and ATTO 633) and the free fluorophore (Cy5 and Green Rhodamine). For the ACF of PEI and siRNA alones we used a 2-component fitting. Finally, the calculated diffusion times were used for a 3-component fitting of the ACF_G-PEI and ACF_R-siRNA resulting from the FCCS experiment of the polyplex, that leads to the fractions of the different components, which were necessary to calculate the number of molecules.

As first step it was necessary calibrate the effective volumes (V_{eff}) with an appropriate fluorophore of know diffusion coefficient[1] for each wavelength (Rhodamine 123 and ATTO 633 with 440 and 340 μm^2/s, respectively).

In the case of the siRNA, we fitted the ACF of siRNA alone with 2-component fitting, keeping the diffusion time of the Cy5 (τ_1) fixed[1] in order to obtain the siRNA one (τ_2). Then we fixed τ_2 in the analysis of the ACF_R-siRNA (3-component fitting) to determinate the diffusion time of the polyplexes (τ_3) and the fraction of each specie (ρ_1, ρ_2, ρ_3). From this analysis, we confirmed the hypothesis of no free siRNA in solution since its fraction was cero ($\rho_2 = 0$).

In the case of PEI, the analysis was quite similar, but it was necessary to fit the Green Rhodamine alone in order to calculate the unknown diffusion time (τ'_1). Furthermore, in the analysis of the ACF_G-PEI, we kept fixed the diffusion time of the polyplexes[1] (τ'_3), obtained from the siRNA analysis, leading to the fraction of free PEI (ρ'_2).

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[1]Since the effectives volumes of each wavelength are different, we fixed the τ'_3 in order to obtain the same diffusion coefficient as in ACF_R-siRNA fitting. In addition, to calculate $N_{F,PEI}$ we used the concentration obtained from FCS analysis of ACF_G-PEI and multiply it by V_{eff} obtained from the calibration with ATTO 633 and the Avogadro's number.

Moreover, to estimate the effective N/P ratio is necessary to know the values of both, the total number of PEI ($N_{T,PEI}$) and siRNA ($N_{T,siRNA}$) molecules in the effective volume (V_{eff}). We can estimate them from the concentrations used to form polyplexes[2] (C_{PEI}, C_{siRNA}) multiplied them by V_{eff} and Avogadro's number (N_A). At the same time, they are equal to the amount of free siRNA and PEI plus the ones in the polyplexes.

$$N_{T,PEI} = C_{PEI} \, V_{eff} \, N_A = N_{F,PEI} + n_{P,PEI} N_{PP}$$

Eq. S2

$$N_{T,siRNA} = C_{siRNA} \, V_{eff} \, N_A = N_{F,siRNA} + n_{P,siRNA} N_{PP}$$

Eq. S3

Where $N_{F,PEI}$ and $N_{F,siRNA}$ are the number of free PEI and siRNA. The $n_{P,PEI}$ and $n_{P,siRNA}$ are the number of PEI and siRNA per polyplex and N_{PP} is the total number of polyplexes obtained from the ACF_R-siRNA. Since we know the fraction of free PEI (ρ'_2) from the ACF_G-PEI we can calculate $N_{F,PEI}$ and $n_{P,PEI}$ from Eq. S2. On the other hand, as was confirmed the total amount of siRNA was in the polyplexes, hence $N_{F,siRNA} = 0$, therefore $n_{P,siRNA}$ is calculate from Eq. S3 dividing $N_{T,siRNA}$ by N_{PP}.

Finally, we can estimate the effective N/P ratio as usual, multiplying the number of primary protonable amino groups (187) of the polymer by the number of PEI molecules per polyplex and divided them by the number of phosphates of the backbone (42) multiplied by the number of siRNA molecules per polyplex.

$$N/P_{eff} = \frac{187 \, n_{P,PEI}}{42 \, n_{P,siRNA}}$$

Eq. S4

Since we had free PEI is expected that the effective N/P ratio is lower than the theorical one. The results are present in the following table (Table S3).

Table S3: Values of the number of siRNA and PEI molecules per polyplex and their respective effective N/P ratio for each theorical one. These values are the average of three measurements of each N/P ratio and the error is the standard deviation of them.

N/P_{Theo}	N_{PP}	$n_{P,siRNA}$	$n_{P,PEI}$	N/P_{eff}
2	6.8 ± 0.8	15 ± 2	5.2 ± 0.7	1.5 ± 0.1
4	3.3 ± 0.5	16 ± 2	11 ± 1	3.1 ± 0.2
12	2.2 ± 0.2	3.8 ± 0.4	6.2 ± 0.8	7.1 ± 0.2

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[2] We used a fixed amount of PEI (135 nM) and different concentrations of siRNA (50, 150 and 300 nM) to obtain the desired N/P ratio (12, 4 and 2, respectively).