Supporting Information

Structure and dynamics of thermosensitive pDNA polyplexes studied by time-resolved fluorescence spectroscopy

Lies A.L. Fliervoet[1], Ekaterina S. Lisitsyna[2], Nikita A. Durandin[2], Ilias Kotsis[1], Roel F.M. Maas-Bakker[1], Marjo Yliperttula[3], Wim E. Hennink[1], Elina Vuorimaa-Laukkanen[2]*, and Tina Vermonden[1]*

[1] *Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, PO Box 80082, 3508 TB Utrecht, the Netherlands*

[2] *Department of Chemistry and Bioengineering, Tampere University of Technology, PO Box 541, FI-33101 Tampere, Finland*

[3] *Division of Pharmaceutical Biosciences and Drug Research Program, P.O. Box 56 (Viikinkaari 5E), University of Helsinki, Finland*

Materials and Methods for Supporting Figures S5, 15 and 16

Materials

Poly(ethylene glycol) (PEG, 6 kDa) and poly(ethylene glycol) bis[2-(dodecylthiocarbonothioylthio)-2-methylpropionate] (CTA-PEG-CTA, 10 kDa) were obtained from Sigma-Aldrich.

Control polymerization reactions

To evaluate whether ATRP can be carried out in the presence of the RAFT initiator, without initiating or disrupting the RAFT initiator, control reactions were performed. In short, ATRP of NIPAM was performed in a mixture of two initiators (Br-PEG-Br and CTA-PEG-CTA). Subsequently, the product of this reaction was subjected to conditions used in the RAFT polymerization of DMAEMA. An overview of the various reaction conditions is shown in Table S1.

1. ATRP polymerization of NIPAM

Triblock copolymers consisting of PNIPAM-PEG-PNIPAM were synthesized following a previously reported procedure using the PEG macroinitiator ($(Br\text{-}C(CH_3)_2\text{-}COO)_2\text{-}PEG_{6000}$).[1] In brief, the PEG macroinitiator (1 equiv.), NIPAM (302 equiv.), CuBr (1 equiv.) and $CuBr_2$ (1 equiv.) were placed in an airtight screw-cap glass vial and 12 mL of H_2O was added to yield a final NIPAM concentration of 133 mg/mL. The reaction mixture was flushed with nitrogen and placed in an ice bath, and Me_6TREN (2 equiv.) was added to start the polymerization reaction, which was carried out for one hour on ice. The polymer solution was dialyzed (MWCO: 10 kDa) against water for 48 hours at 4 °C, while changing the dialysate three times a day and subsequently lyophilized. In addition, the same polymerization reaction was carried out but with the

presence of the commercially available CTA-PEG-CTA macroinitiator as well (mixture B-1). All products were analyzed by GPC as described in section 2.5.2 of the manuscript.

2. RAFT polymerization of DMAEMA

To assess if the RAFT macroinitiator is still able to initiate the polymerization of DMAEMA, after ATRP, the polymer products of reactions A-1 and B-1 were dissolved in dry DMF and AIBN was added (reaction conditions were similar as described in section 2.3.2 of the manuscript). At least three freeze-pump-thaw cycles were applied to degas the solution, after which the reaction mixture was placed in an oil bath at 70 °C and stirred for 16 hours under N_2 atmosphere. The polymer solution was transferred into a dialysis cassette (MWCO: 10 kDa) and dialyzed against water for 48 hours at 4 °C, while changing the dialysate three times a day. The final polymers was recovered by freeze drying and analyzed by GPC as described in section 2.5.2 of the manuscript.

Table S1. Overview of control polymerization reactions performed under ATRP conditions (mixture A1 and B-1) or RAFT conditions (mixture A-2 and B-2).

Reference	Polymerization reaction	Substances	Expected products
Mixture A-1	ATRP	Br-PEG-Br + NIPAM	PNIPAM-PEG-PNIPAM
Mixture B-1	ATRP	Br-PEG-Br + CTA-PEG-CTA + NIPAM	PNIPAM-PEG-PNIPAM + CTA-PEG-CTA
Mixture A-2	RAFT	Product A-1 + DMAEMA	PNIPAM-PEG-PNIPAM
Mixture B-2	RAFT	Product B-1 + DMAEMA	PNIPAM-PEG-PNIPAM + PDMAEMA-PEG-PDMAEMA

Dynamic light scattering (DLS) and Laser Doppler electrophoresis (LDE) analysis of polyplexes

DLS was used to determine the hydrodynamic size and polydispersity index (PDI) of the formed polyplexes. Samples were measured in HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) at a final pDNA concentration of 20 µg/mL. The measurements were performed on a Zetasizer Nano S (Malvern Instruments, Malvern, UK) with an He-Ne laser operating at 633 nm, and temperature controller set at 4 or 37 °C. Data were corrected for viscosity at the different temperatures using the Malvern Zetasizer software (version 7.12). The ζ-potential of the polyplexes was measured using laser Doppler electrophoresis on a Zetasizer Nano Z

(Malvern Instruments). Samples were measured in HEPES buffer (20 mM HEPES, pH 7.4) at a final pDNA concentration of 20 µg/mL.

Supporting Figures

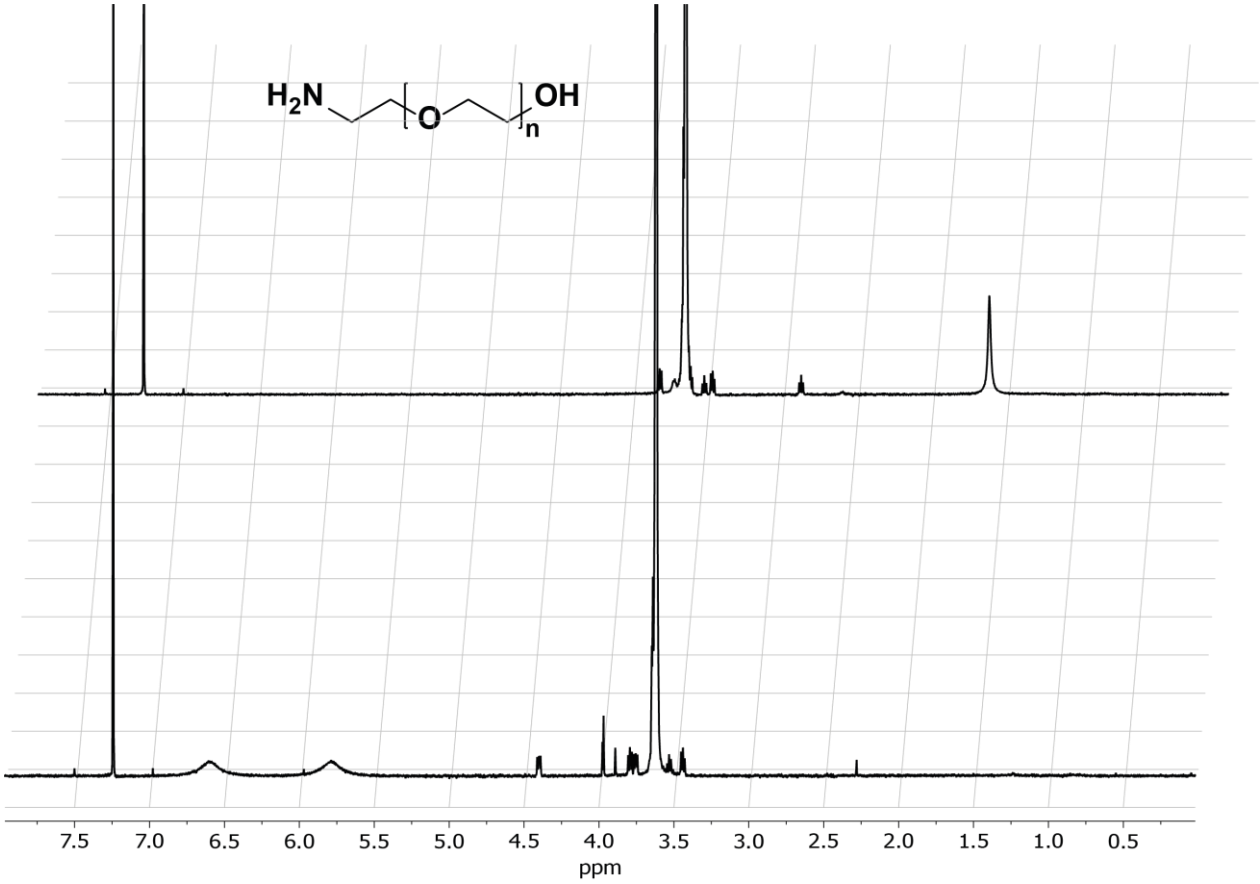

Figure S1. Top: ^{1}H-NMR spectrum of NH_2-PEG-OH in $CDCl_3$. δ (ppm) 3.8-3.5 (478H, $(\boldsymbol{CH_2})_2O$). Bottom: ^{1}H-NMR spectrum of NH_2-PEG-OH in $CDCl_3$ + TAIC. Reaction of TAIC with the terminal hydroxyl groups caused a shift of the CH_2 protons of PEG adjacent to the OH end to 4.4 ppm.

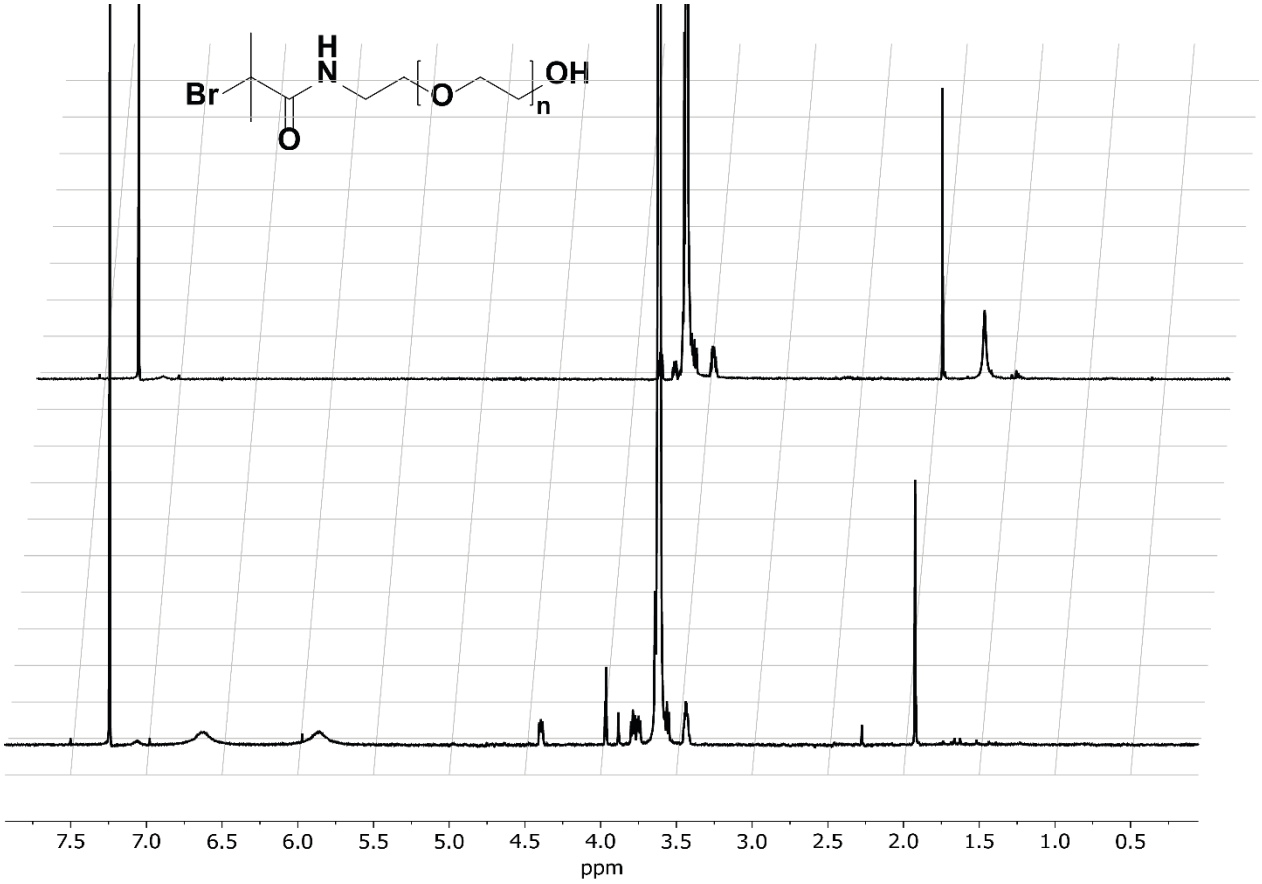

Figure S2. Top: ^{1}H-NMR spectrum of Br-C$(CH_3)_2$-CO-NH-PEG-OH in $CDCl_3$. δ (ppm) 3.8-3.5 (478H, (***CH₂***)$_2$O), 1.94 (6H, (***CH₃***)$_2$CBr). Bottom: ^{1}H-NMR spectrum of Br-C$(CH_3)_2$-CO-NH-PEG-OH in $CDCl_3$ + TAIC. Reaction of TAIC with the terminal hydroxyl groups caused a shift of the CH_2 protons of PEG adjacent to the OH end to 4.4 ppm.

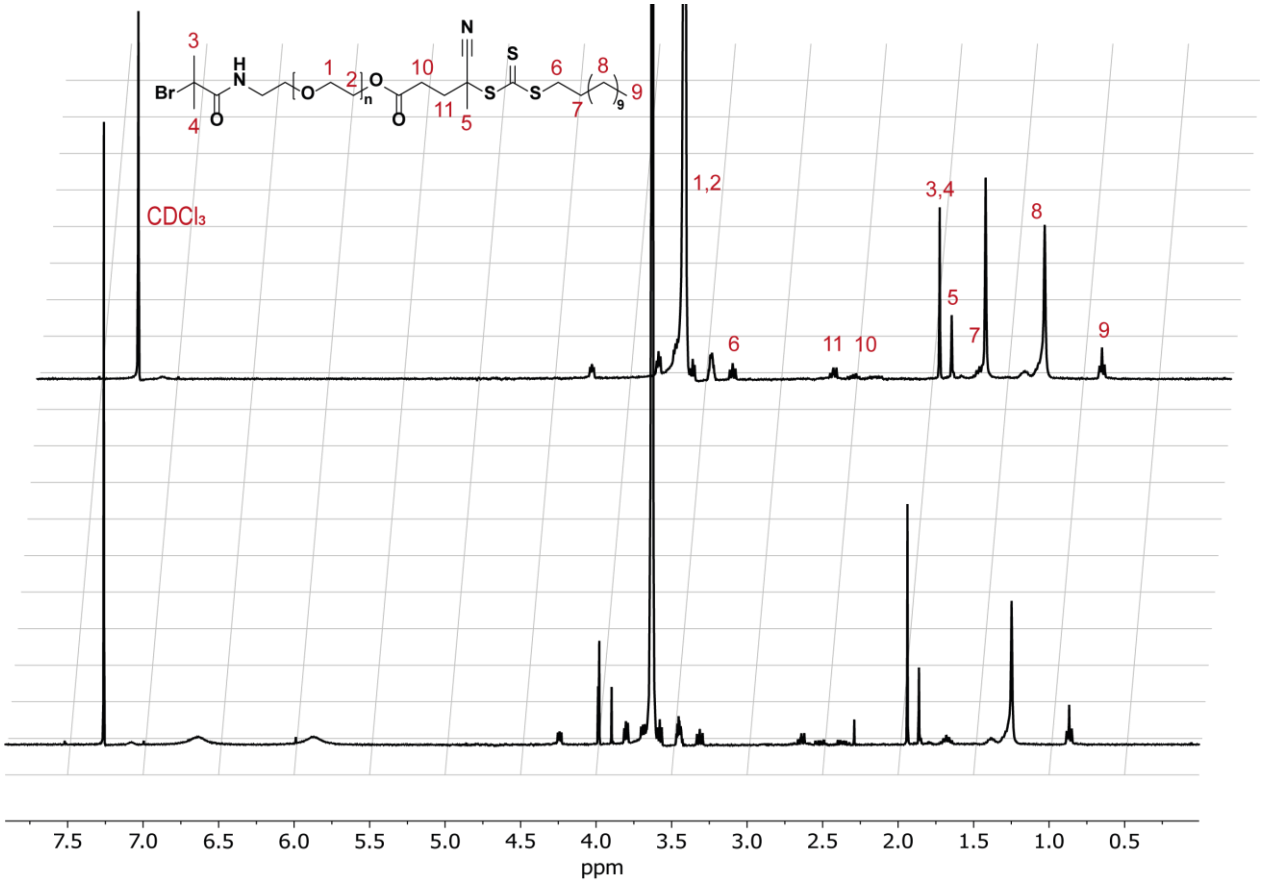

Figure S3. Top: ^{1}H-NMR spectrum of Br-C$(CH_3)_2$-CO-NH-PEG-CTA in $CDCl_3$. δ (ppm) 3.8-3.5 (478H, O(***CH₂***)$_2$O), 3.32 (2H, S(C=S)S***CH₂***), 2.75 (2H, ***CH₂***$(CH_2)_9CH_3$), 2.65 (2H, (C=O)CH_2***CH₂***C(CN)), 2.52-2.37 (2H, (C=O)***CH₂***CH_2C(CN)), 1.94 (6H, (***CH₃***)$_2$CBr), 1.88 (3H, ***CH₃***C(CN)), 1.41-1.22 (18H, CH_2(***CH₂***)$_9CH_3$), 0.88 (3H, $CH_2(CH_2)_9$***CH₃***). Bottom: ^{1}H-NMR spectrum of Br-C$(CH_3)_2$-CO-NH-PEG-CTA in $CDCl_3$ + TAIC.

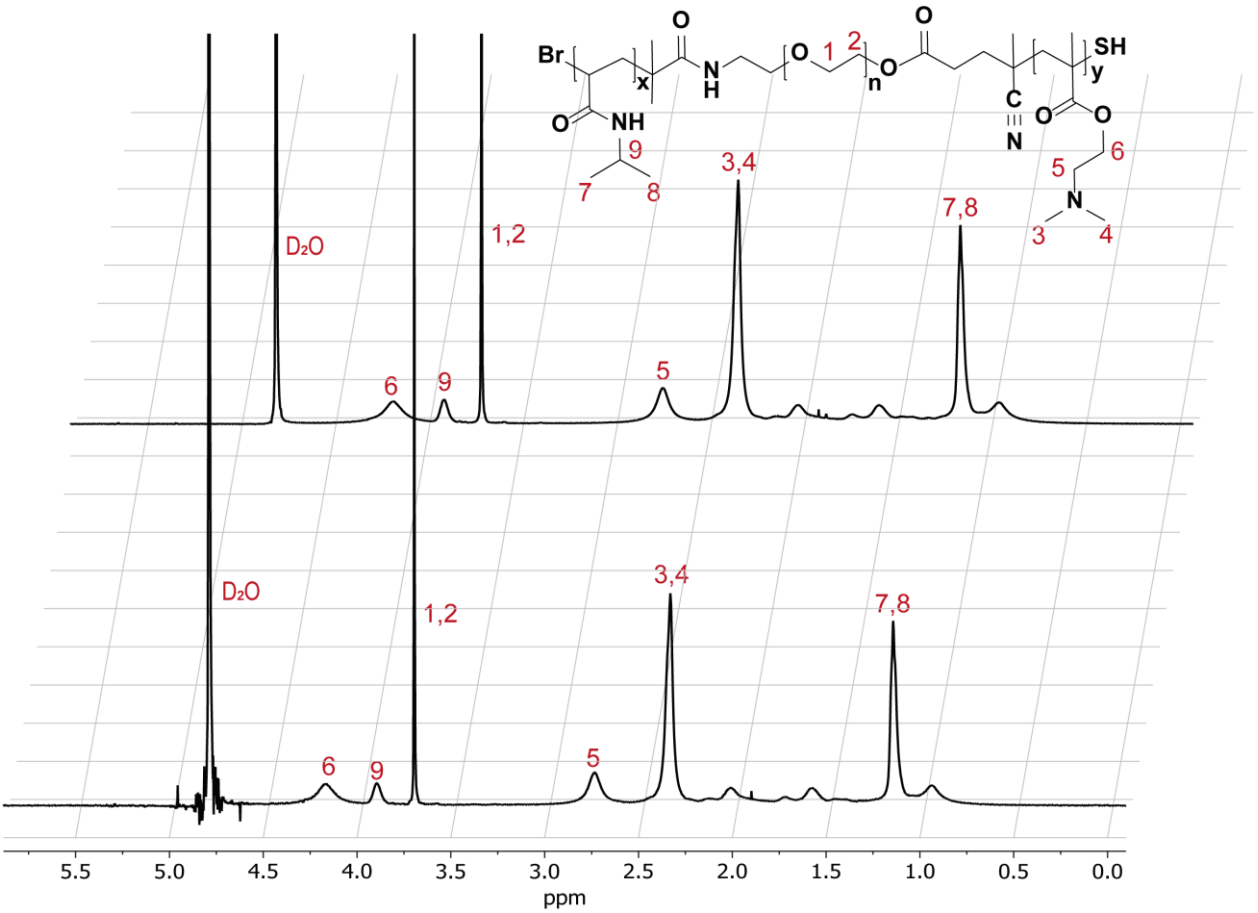

Figure S4. Top: ^{1}H-NMR spectrum of NPD polymer in D_2O. Bottom: ^{1}H-NMR spectrum of Cy3-labeled NPD polymer in D_2O.

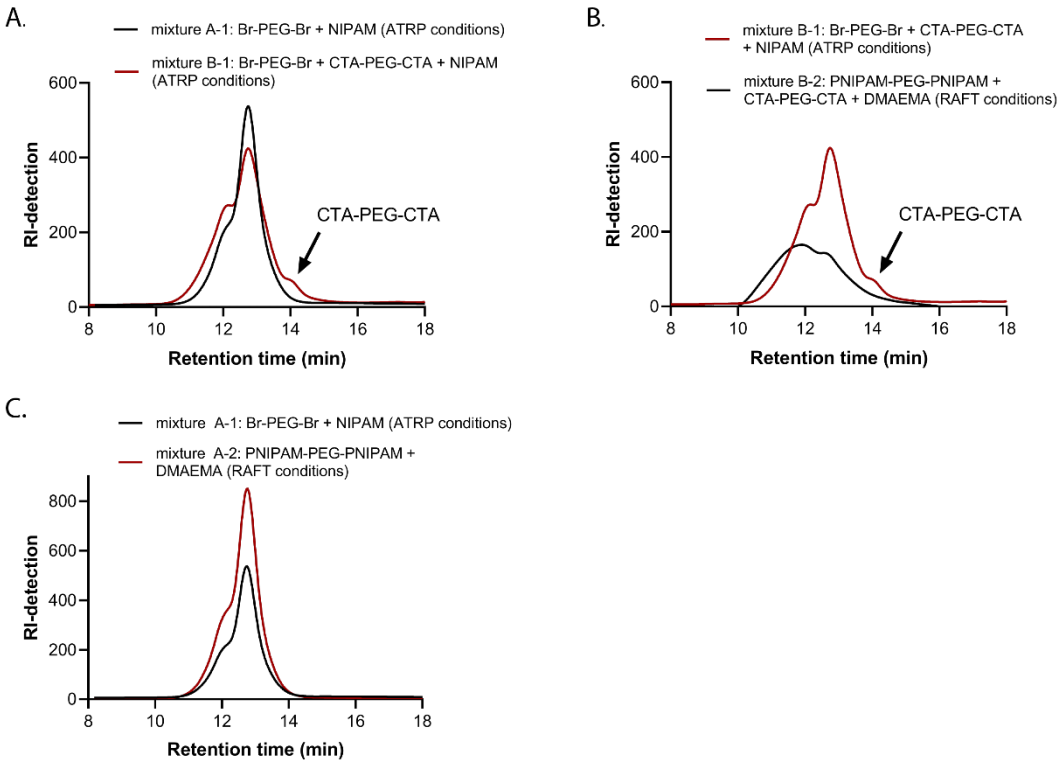

Figure S5. GPC chromatograms of control polymerization reactions.

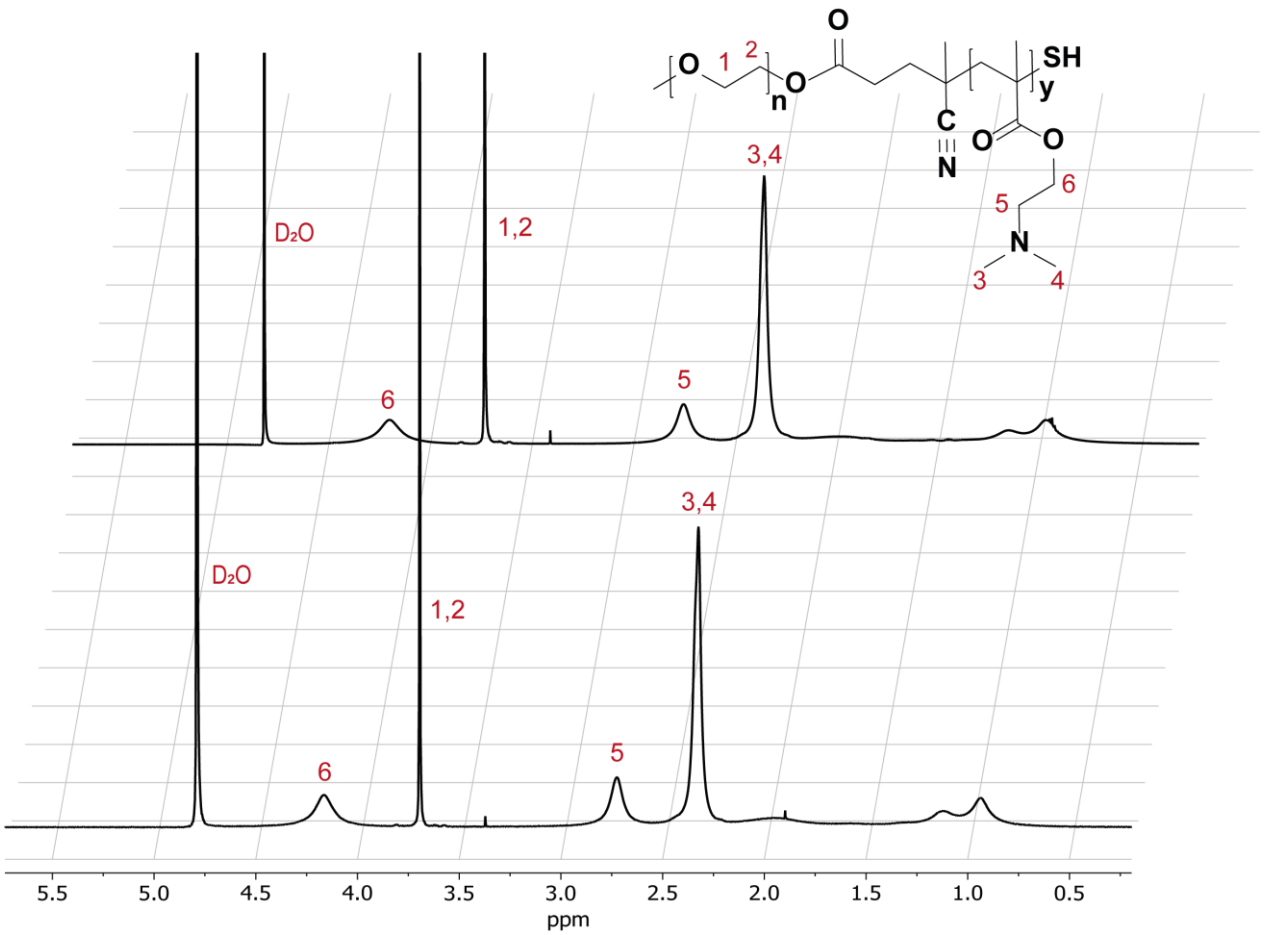

Figure S6. Top: ^{1}H-NMR spectrum of PD polymer in D_2O. Bottom: ^{1}H-NMR spectrum of Cy3-labeled PD polymer in D_2O.

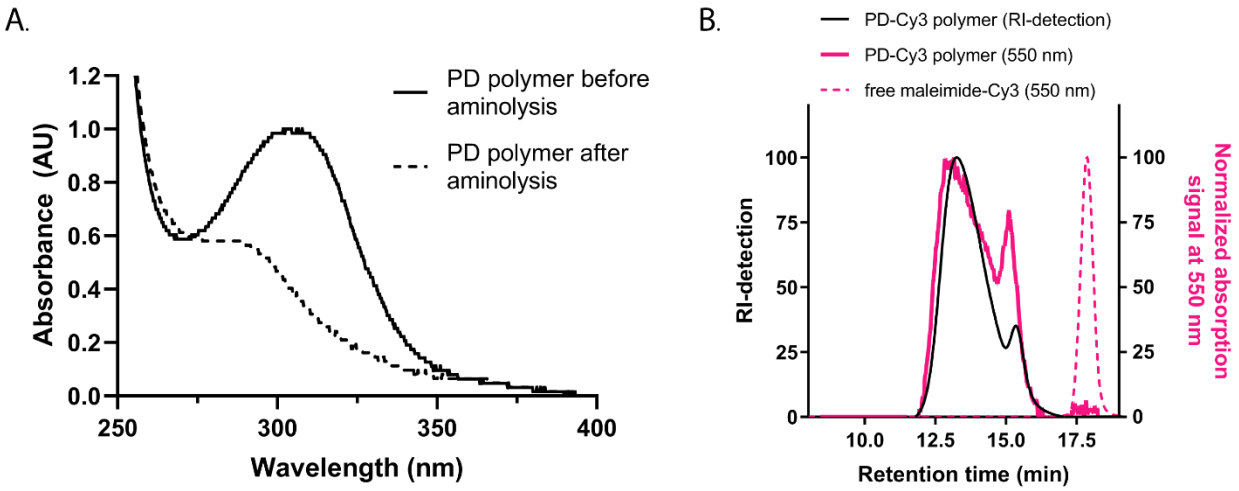

Figure S7. A) UV-vis spectra of PD polymer before (solid line) and after (dotted line) aminolysis with *n*-butylamine for 24 hours at RT. B) GPC analysis with dual RI (black) and UV-vis (at 550 nm, pink) detection of Cy3-labeled PD polymer (solid line) and free maleimide-Cy3 dye (dashed line).

Table S2. Characteristics of NPD triblock and PD diblock copolymers synthesized by radical polymerization. The polymer names are abbreviated according to the block composition (N = PNIPAM, P = PEG, D = PDMAEMA).

Name	Free -SH (%)[a]	Labeling density (%)[b]
NPD-SH	11	*n.a*
NPD-Cy3	*n.d*	6
PD-SH	7	*n.a*
PD-Cy3	*n.d*	5

[a] Determined by Ellman's assay. [b] Determined by UV-vis spectroscopy. *n.a.* = not applicable, *n.d.* = not determined.

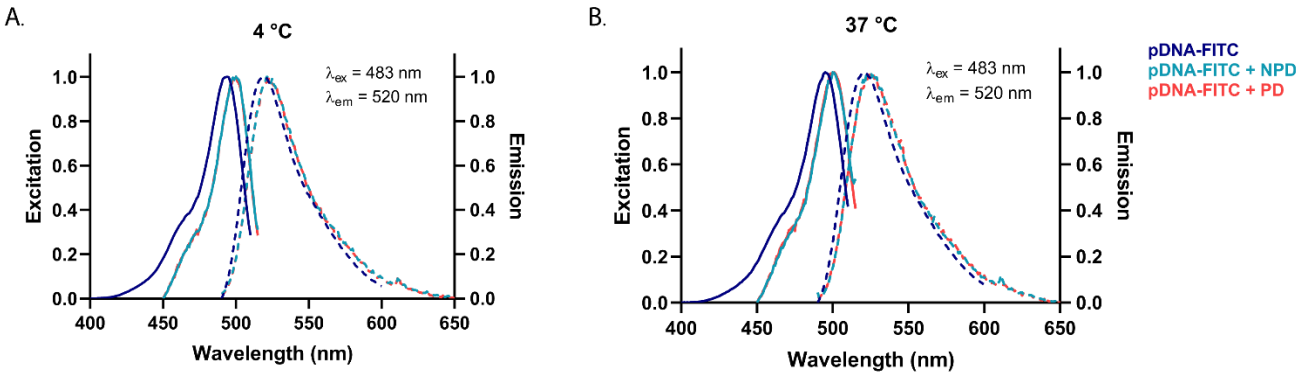

Figure S8. Normalized excitation (solid line) and emission (dotted line) spectra for pDNA-FITC in the presence and absence of unlabeled polymers (NPD and PD) at N/P 10 at 4 (A) and 37 °C (B).

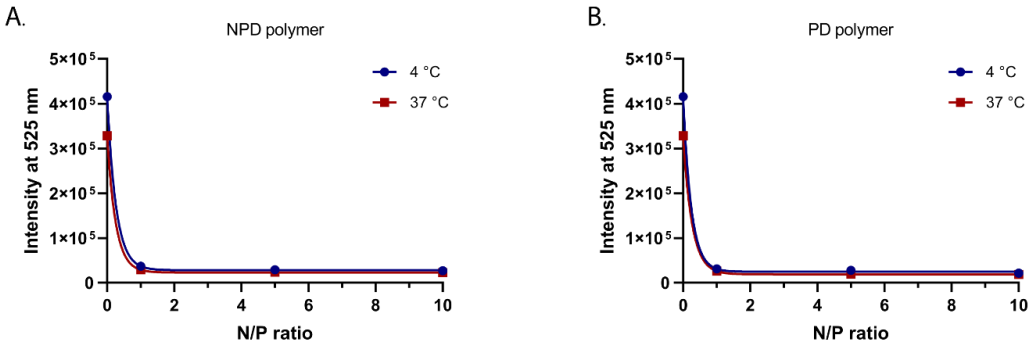

Figure S9. Fluorescence intensity at 520 nm for pDNA-FITC in the presence of NPD (A) and PD (B) polymer at different N/P ratios at 4 and 37 °C.

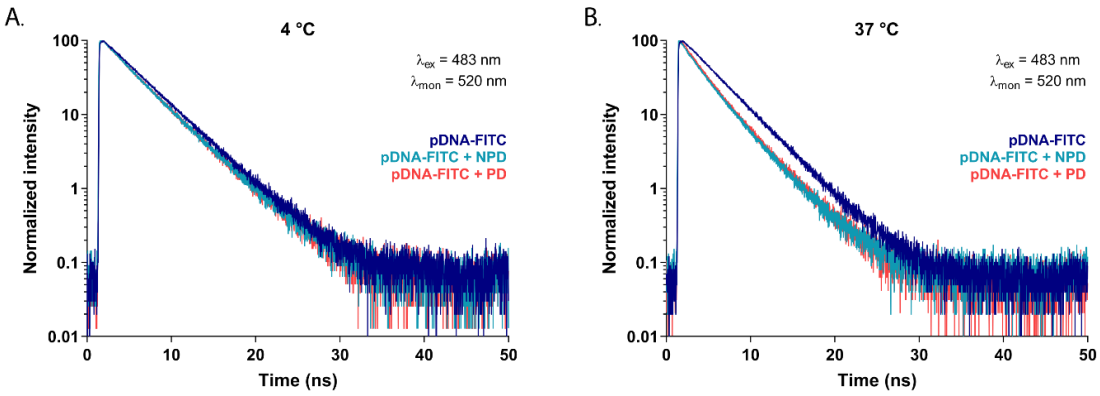

Figure S10. Fluorescence decay curves for pDNA-FITC in the presence and absence of unlabeled polymers (NPD and PD) at N/P 10 at 4 (A) and 37 °C (B). The excitation wavelength was 483 nm and the decays were monitored at 520 nm.

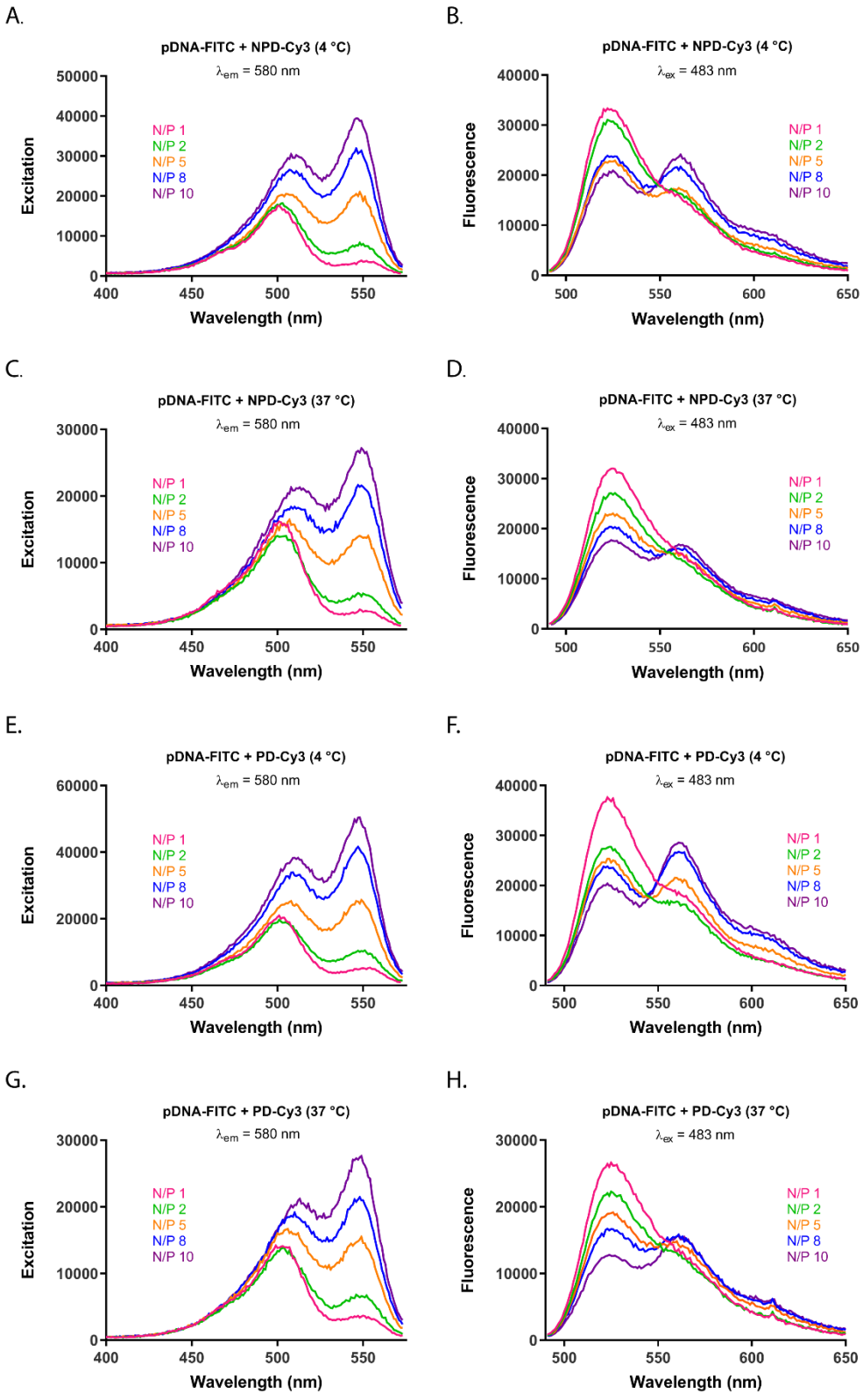

Figure S11. Excitation and fluorescence spectra for pDNA-FITC in the presence of Cy3-labeled polymers (NPD-Cy3 and PD-Cy3) at different N/P ratios at 4 and 37 °C.

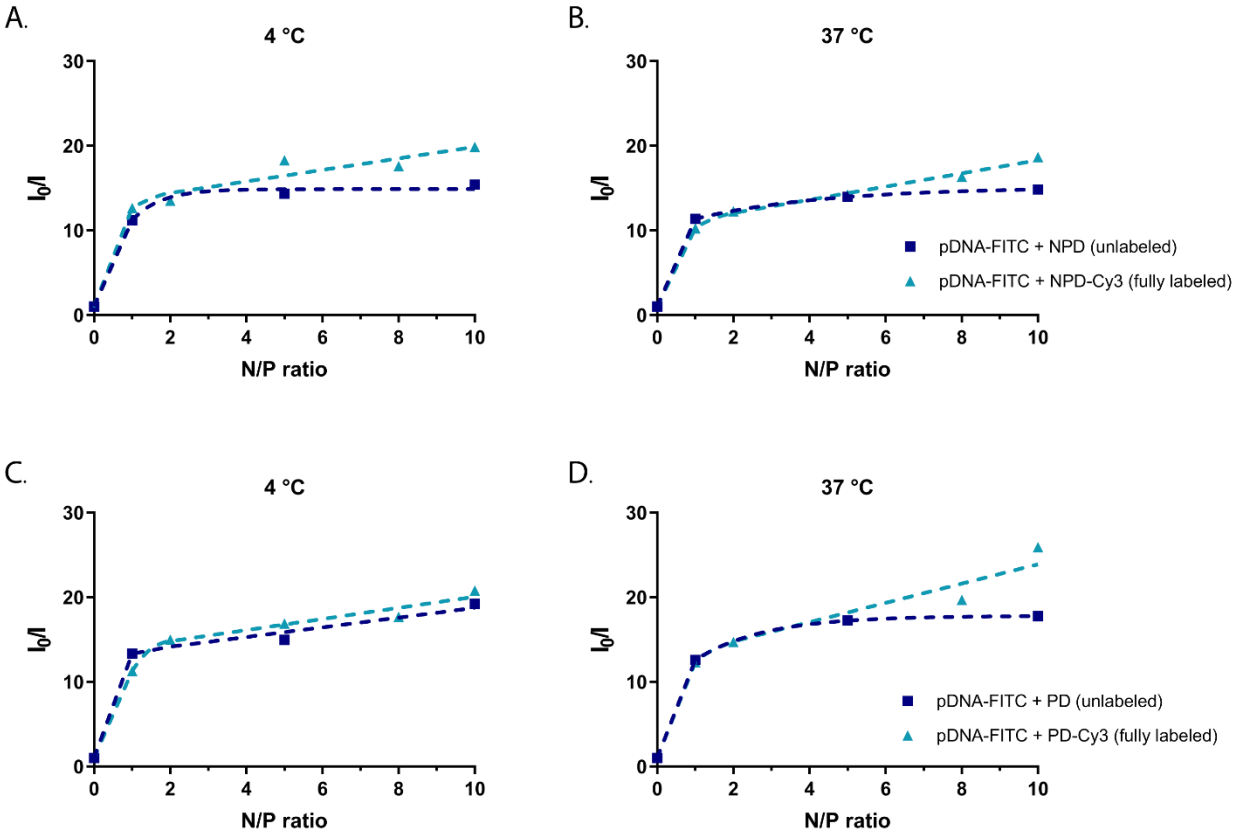

Figure S12. Intensity ratio versus N/P ratio for pDNA-FITC in the presence of unlabeled and Cy3-labeled NPD polymer (A-B) and in the presence of unlabeled and Cy3-labeled PD polymer (C-D) at 4 and 37 °C. The fluorescence intensity of pDNA-FITC (I_0) at 520 nm was used to calculate the fluorescence intensity ratio (I_0/I).

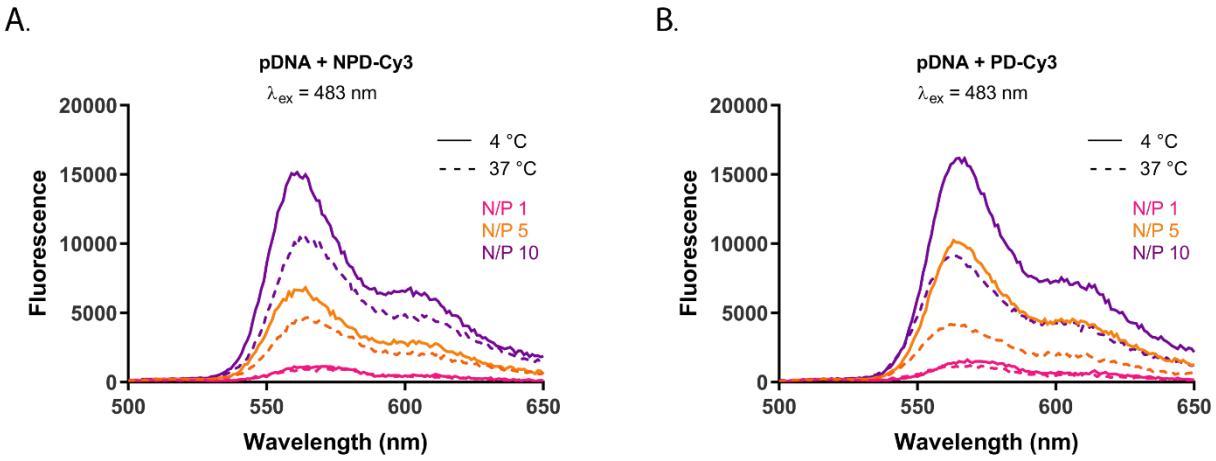

Figure S13. Fluorescence spectra at 4 °C (solid line) and 37 °C (dotted line) for pDNA in the presence of NPD-Cy3 (A) and NP-Cy3 (B) at different N/P ratios.

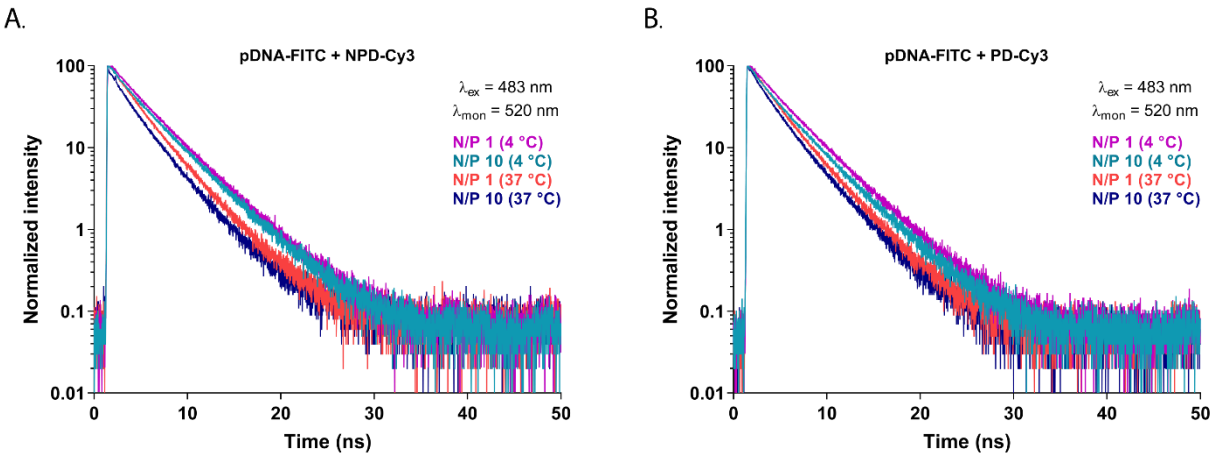

Figure S14. Fluorescence decay curves for pDNA-FITC in the presence of NPD-Cy3 (A) and PD-Cy3 (B) polymers at N/P 1 and 10 at 4 and 37 °C. The excitation wavelength was 483 nm and the decays were monitored at 520 nm.

Table S3. Fluorescence lifetimes (τ_1, τ_2), error of the lifetimes ($d\tau_1$, $d\tau_2$), the proportion of the longer living component (α_1) and the mean amplitude weighted fluorescence lifetime ($\langle\tau\rangle$) at 4 and 37 °C for pDNA-FITC in the presence of labeled polymers (NPD-Cy3 and PD-Cy3).

Sample	Temp. (°C)	N/P ratio	τ_1 (ns)	$d\tau_1$ (ns)	α_1 (%)	τ_2 (ns)	$d\tau_2$ (ns)	$\langle\tau\rangle$ (ns)
pDNA-FITC + NPD-Cy3 *(fully labeled)*	4	1	3.90	0.03	80	1.33	0.13	3.38
		2			78			3.33
		5			76			3.33
		8			74			3.23
		10			71			3.17
	37	1	3.30	0.05	72	1.27	0.10	2.73
		2			68			2.64
		5			63			2.55
		8			60			2.50
		10			57			2.42
pDNA-FITC + PD-Cy3 *(fully labeled)*	4	1	3.84	0.03	83	1.29	0.11	3.41
		2			76			3.23
		5			72			3.13
		8			68			3.03
		10			68			3.02
	37	1	3.40	0.04	67	1.42	0.08	2.75
		2			64			2.69
		5			59			2.60
		8			56			2.54
		10			53			2.48
pDNA-FITC + NPD(-Cy3) *(mixed)*	4	1	3.98	0.04	86	1.52	0.17	3.63
		2			84			3.59
		5			81			3.52
		8			76			3.40
		10			73			3.33
	37	1	3.39	0.05	73	1.43	0.11	2.86
		2			77			2.95
		5			72			2.83
		8			57			2.55
		10			57			2.54
pDNA-FITC + PD(-Cy3) *(mixed)*	4	1	4.05	0.04	83	1.60	0.15	3.63
		2			82			3.61
		5			79			3.53
		8			72			3.37
		10			67			3.25
	37	1	3.44	0.05	69	1.45	0.08	2.82
		2			66			2.75
		5			59			2.63
		8			53			2.51
		10			49			2.42

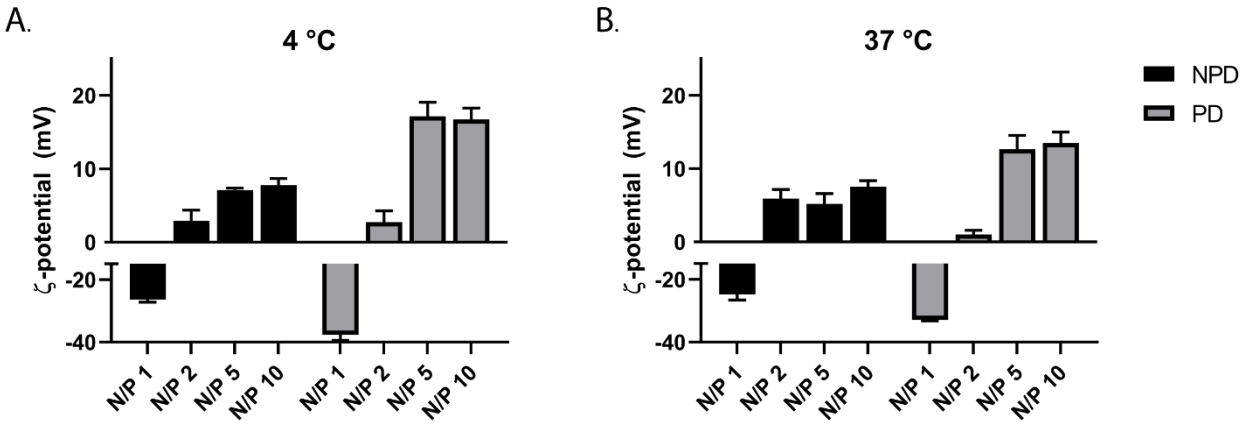

Figure S15. ζ-potential of NPD and PD polyplexes formed and measured at 4 °C (A) or 37 °C (B) by laser Doppler electrophoresis (LDE) in HEPES buffer (20 mM HEPES, pH 7.4).

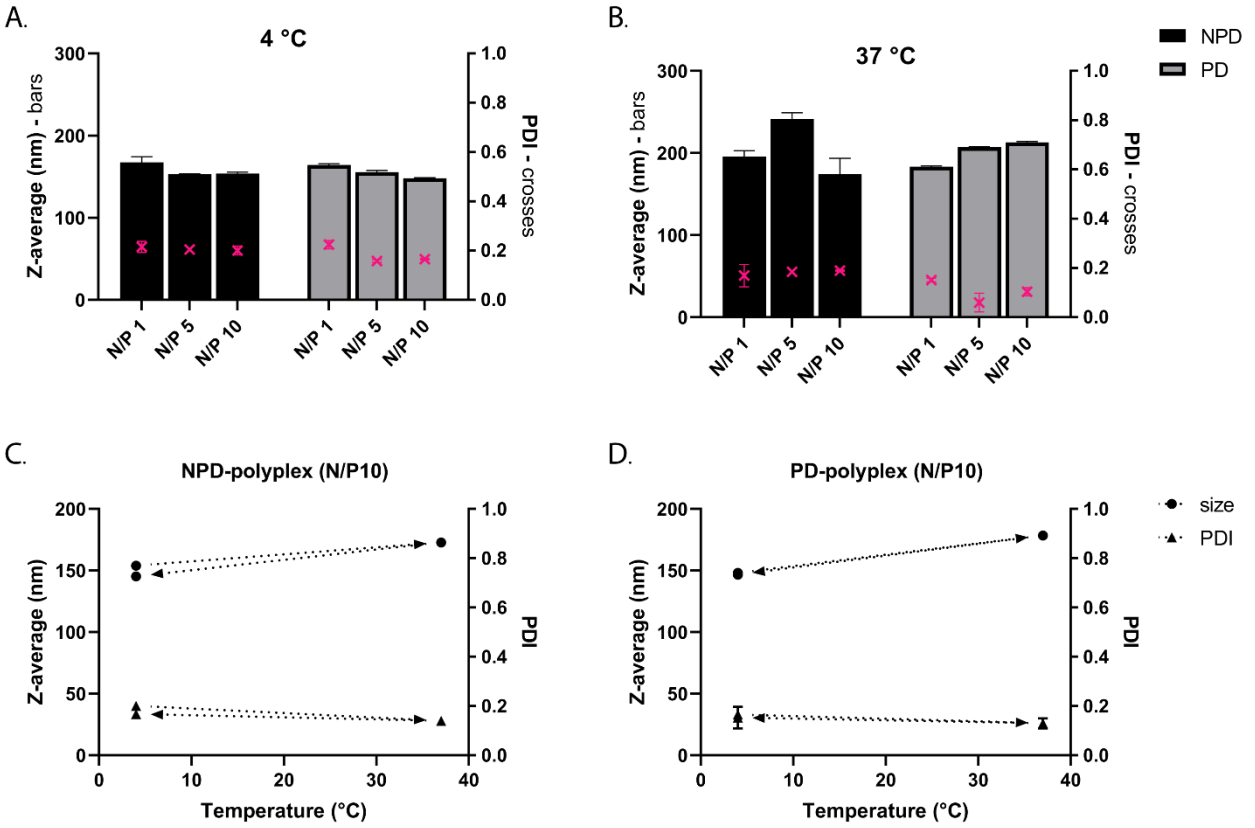

Figure S16. A&B) Particle size (bars) and polydispersity index (PDI, crosses) of NPD and PD polyplexes with various N/P ratios formed and measured at constant temperature of 4 °C or 37 °C. C&D) Particle size (dots) and PDI (triangles) of NPD and PD polyplexes with N/P 10 formed and measured at 4 °C and subjected to a temperature change 37 and back to 4 °C (direction of the temperature change is indicated with the arrows). All samples are measured by dynamic light scattering (DLS) in HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4).

Table S4. Fluorescence lifetimes (τ_1, τ_2), error of the lifetimes ($d\tau_1$, $d\tau_2$), the proportion of the longer living component (α_1) and the mean amplitude weighted fluorescence lifetime ($<\tau>$) for pDNA-FITC in the presence of unlabeled polymers (NPD and PD) for temperature cycle measurements. Results are obtained from 2-exponential fitting, and values marked in red correspond to corrupted fitting values.

Sample	Temp. (°C)	N/P ratio	τ_1 (ns)	$d\tau_1$ (ns)	α_1 (%)	τ_2 (ns)	$d\tau_2$ (ns)	$<\tau>$ (ns)
pDNA-FITC + NPD	4	10	4.03	0.05	74	1.51	0.18	3.38
	22		3.69	0.05	72	1.18	0.15	2.99
	37		3.27	0.05	65	0.66	0.13	2.35
	22		3.59	0.05	71	0.80	0.15	2.79
	4		3.78	*n.a*	70	0.43	*n.a*	2.77
	37		3.22	0.04	71	0.66	0.14	2.49
	22		3.65	0.05	73	1.22	0.17	2.99
	4		3.91	0.05	77	1.16	0.20	3.27
	22		3.53	0.04	74	0.74	0.16	2.80
	37		3.19	0.06	65	0.65	0.14	2.29
pDNA-FITC + PD	4	10	3.86	0.06	75	0.83	0.19	3.09
	22		3.68	0.07	72	1.14	0.21	2.98
	37		3.40	0.07	67	1.06	0.16	2.64
	22		3.60	0.06	70	0.86	0.17	2.78
	4		3.83	0.05	74	0.67	0.17	3.00
	37		3.39	0.07	65	1.20	0.16	2.63
	22		3.58	0.06	73	0.92	0.19	2.86
	4		3.80	0.05	74	0.65	0.17	2.98
	22		3.54	0.05	72	0.70	0.17	2.76
	37		3.36	0.07	65	0.94	0.16	2.51

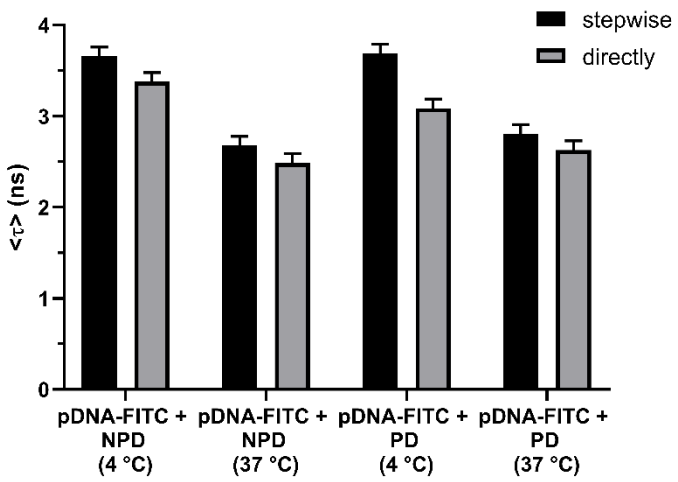

Figure S17. Average fluorescence lifetimes for NPD- and PD-based polyplexes prepared either stepwise or directly at N/P 10 at 4 and 37 °C.

Reference

1. de Graaf, A. J.; Azevedo Prospero dos, S., II; Pieters, E. H.; Rijkers, D. T.; van Nostrum, C. F.; Vermonden, T.; Kok, R. J.; Hennink, W. E.; Mastrobattista, E., A micelle-shedding thermosensitive hydrogel as sustained release formulation. *Journal of Controlled Release* **2012,** *162* (3), 582-90.