Supporting Information

Core-crosslinked, temperature- and pH-responsive micelles: Design, physicochemical characterization, and gene delivery application

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1 Synthesis of PDSAc

Synthesis of 2-(Pyridin-2-yldisulfanyl)ethyl acrylate (PDSAc)

PDSAc was synthesized according to an adapted procedure from literature.[1]

2-(Pyridin-2yldisulfanyl)ethanol (PDS-OH)

2,2′-Dipyridyl disulfide (11.40 g, 51.7 mmol) was dissolved in 100 mL dry methanol in a 500 mL two-neck round-bottom flask equipped with a magnetic stirring bar. Then glacial acetic acid (0.74 mL, 12.93 mmol) was added to the solution. Afterward, 2-mercaptoethanol (4.0 mL, 56.83 mmol) was dissolved in 50 mL methanol in a pressure-equalizing dropping funnel and was added dropwise at room temperature over 1 h to the solution. Following complete addition, the green solution was left to stir at room temperature overnight. Subsequently, the solvent was removed under reduced pressure to yield a yellow viscose oil. The crude product was dissolved in 200 mL of a mixture of *n*-hexane/ethyl acetate (50/50 v/v) and the formed, green precipitate was filtered off. Afterward, the solvent was removed under reduced pressure and the crude product was purified *via* flash chromatography (silica, *n*-hexane/ethyl acetate 50/50 v/v with a gradient, $R_f = 0.25$) to yield PDS-OH as a yellow oil (5.22 g, 27.87 mmol, 54 %).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.87 (t, 2H, −S−S−C***H*****$_2$**−), 3.73 (t, 2H, −C***H*****$_2$**−OH), 5.70 (s, 1H, −O***H***), 7.06 (t, 1H, −C***H***=CH−N=C(CH−)−S−), 7.38 (d, 1H, −C***H***−(C=N−)−S−), 7.52 (t, 1H, −C***H***=CH−(C=N−)−S−), 8.41 (d, 1H, −CH=C***H***−N=C(CH−)−S−) ppm.
^{13}C NMR (75 MHz, $CDCl_3$): δ = 42.7 (−S−S−***C***H_2−), 58.6 (−***C***H_2−OH), 121.6 (−***C***H=CH−N=C(CH−)−S−), 121.8 (−***C***H−(C=N−)−S−), 137.1 (−***C***H=CH−(C=N−)−S−), 149.9 (−CH=***C***H−N=C(CH−)−S−), 159.4 (−N=***C***(CH−)−S−) ppm.

2-(Pyridin-2-yldisulfanyl)ethyl acrylate (PDSAc)

PDS-OH (5.22 g, 27.87 mmol) was dissolved in 100 mL dry DCM in a 500 mL two-neck round-bottom flask equipped with a magnetic stirring bar and pressure-equalizing dropping funnel, cooled in an ice bath before TEA (4.70 mL, 33.72 mmol) was added. Then acryloyl chloride (2.00 mL, 24.75 mmol) dissolved in 50 mL dry DCM was added over 30 min to the cooled solution. The reaction mixture was left to stir overnight at room temperature. The solution was precipitated twice into -80 °C cold diethyl ether (2 × 200 mL). The precipitate was filtered off and the solvent was removed under reduced pressure. The crude product was purified *via* flash chromatography (silica, *n*-hexane/ethyl acetate 70/30 with a gradient, $R_f = 0.5$) to yield PDSAc as a yellow oil (5.05 g, 20.93 mmol, 75 %).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.06 (t, 2H, −S−S−C***H₂***−), 4.38 (t, 2H, −C***H₂***−O−(C=O)−), 5.83 (dd, 1H, C***H₂***=CH−(C=O)−O−), 6.08 (dd, 1H, CH_2=C***H***−(C=O)−O−), 6.41 (dd, 1H, C***H₂***=CH−(C=O)−O−), 7.06 (t, 1H, −C***H***=CH−N=C(CH−)−S−), 7.64 (m, 2H, −C***H***−(C=N−)−S−, −C***H***=CH−(C=N−)−S−), 8.43 (d, 1H, −CH=C***H***−N=C(CH−)−S−) ppm.
^{13}C NMR (75 MHz, $CDCl_3$): δ = 36.8 (−S−S−***C***H_2−), 61.7 (−***C***H_2−O−(C=O)−), 119.3 (−***C***H=CH−N=C(CH−)−S−), 120.3 (−***C***H−(C=N−)−S−), 127.5 (***C***H_2=CH−), 130.7 (CH_2=***C***H−(C=O)−), 136.5 (−***C***H=CH−(C=N−)−S−), 149.2 (−CH=***C***H−N=C(CH−)−S−), 159.1 (−N=***C***(CH−)−S−), 165.2 (−(***C***=O)−O−) ppm.

HR-ESI-MS: m/z = 242.0311 ($[M+H]^+$) (found), 242.0304 ($[M+H]^+$) (calculated).

2 Calculations for RAFT Polymerization

The monomer conversion (p) was calculated from ^{1}H NMR data by comparing the integrals of vinyl peaks (5.5-5.75 ppm) against the external reference 1,3,5-trioxane (5.10 ppm) before and after polymerization. The theoretical number-average molar mass ($M_{n,th}$) was calculated with eq. S1:

$$M_{\mathrm{n,th}} = \frac{[M]_0 p M_{\mathrm{M}}}{[CTA]_0} + M_{\mathrm{CTA}} \qquad (eq.S1)$$

$[M]_0$ and $[CTA]_0$ are the initial concentrations of monomer and chain transfer agent (CTA), respectively. M_M and M_{CTA} are the molecular masses of the monomer and CTA, respectively.

3 Determination of the degree of crosslinking

A sample of the deprotected polymer pPDA was dissolved in 150 mM NaCl solution to a sample concentration of 4 mg mL^{-1}. Then, 2 mL of the polymer solution was added into each of 10 vials equipped with a magnetic stirring bar, which were then sealed with a cap and transferred to a thermostated oil bath set at 50 °C. The vials were equilibrated for 30 min and equivalents ranging from 0.1 to 1.0 of a 4 mg mL^{-1} solution of 1,6-hexanedithiol in DMSO, regarding the PDSAc units in one polymer chain, were added to each polymer solution respectively. Subsequently, the vials were heated for 1 h and then the vial was cooled to room temperature. Then 1.5 mL of the sample was transferred to a Hellma quartz cuvette and the absorbance of each sample was measured at room temperature from 300 to 450 nm with a Specord 250 UV-Vis spectrophotometer (slit: 2 nm, $\Delta\lambda$ = 0.2 nm, speed = 2 nm s^{-1}). The absorbance of the leaving group 2-thiopyridone of each sample was plotted against the wavelength to find the equivalents of crosslinker, which lead to the highest absorbance and thus identify the maximum amount of PDSAc moieties that are potentially crosslinked (see Fig. S9).

4 Variation of sizes in DLS

The cumulants analysis of DLS autocorrelation functions leads to z-average hydrodynamic size estimates, $d_{h,z}$, based on the Stokes-Einstein equation:

$$d_{h,Z} = \frac{kT}{3\pi\eta_0 D} \quad (eq.S2)$$

where k is the Boltzmann constant, T the absolute temperature, η_0 the solvent viscosity, and D the translational diffusion coefficient. Assuming Gaussian statistics, the standard deviation of sizes of the population (σ) was calculated from the $d_{h,z}$ values and PDI accompanied to the data:

$$PDI = \left(\frac{\sigma}{d_{h,z}}\right)^2 \quad (eq.S3)$$

The size variation within the population is thereby given by $d_{h,z} \pm \sigma$.

5 Ethidium bromide binding assay (EBA) and heparin release assay (HRA)

The pDNA complexation and complex stability was investigated by using an Ethidium bromide (EtBr) quenching and heparin release assay as described elsewhere.[2] Briefly, pKMyc-pDNA at a concentration of 15 µg mL^{-1} was incubated with EtBr in HBG buffer (5 % (w/v)) glucose, 20 mM HEPES (pH 7.4) for 10 min. The material stock solutions were diluted with HBG buffer (pH 7.4) in a black 96-well plate (Nunc, Thermo Fisher, Waltham, MA, U.S.) to reach N/P ratios ranging from 1 of 30. Subsequently, pDNA was added and the complexes were incubated at RT for 15 min. EtBr fluorescence intensity was measured at λ_{Ex} = 525 nm / λ_{Em} = 605 nm. pDNA without polymer was defined as 100 % free pDNA. Afterwards, the polyplexes were incubated at 37 °C for 30 min and the EtBr fluorescence intensity was measured. Subsequently, the release of complexed pDNA at 37 °C was studied by stepwise addition of heparin and measurement of the resulting changes in EtBr fluorescence intensity. The obtained experimental results were plotted, and their mean was fitted by a b-spline function using Origin Pro Software (Version 2018b, Origin Lab Corporation, Northampton, MA, U.S.) to represent the apparent experimental results as a guide to the eye.

6 Additional results

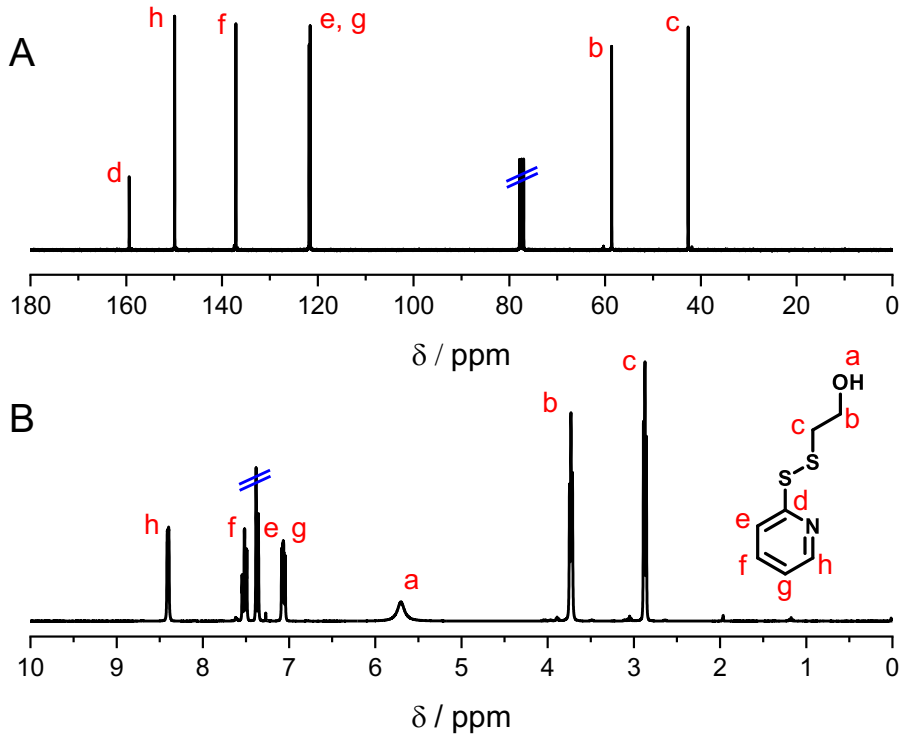

Fig. S1. ^{13}C (**A**) and ^{1}H (**B**) NMR spectra of PDS-OH in $CDCl_3$.

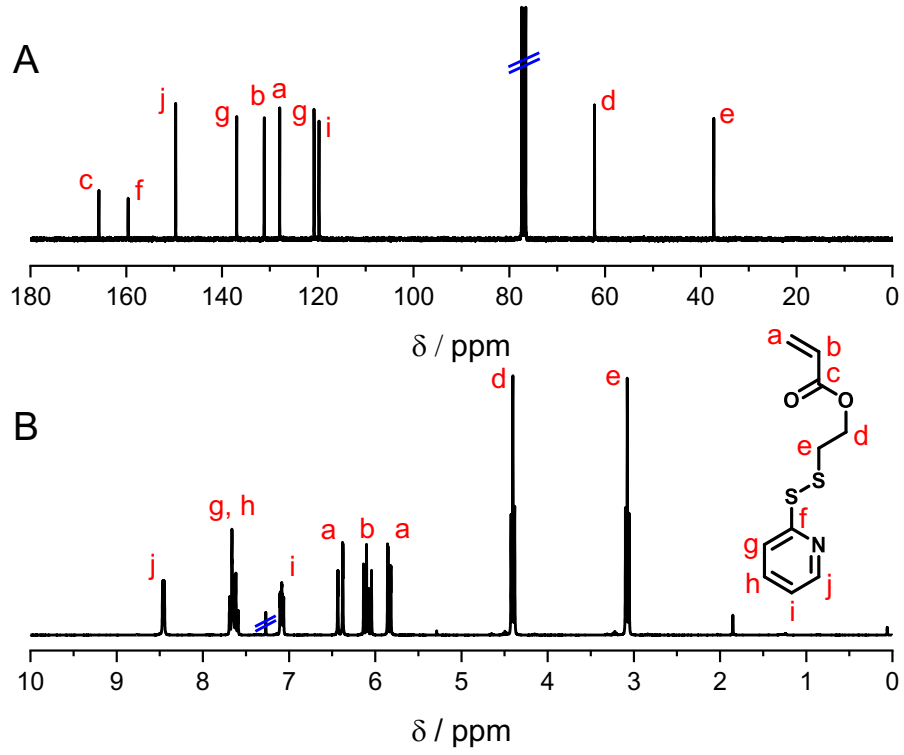

Fig. S2. ^{13}C (**A**) and ^{1}H (**B**) NMR spectra of PDSAc in $CDCl_3$.

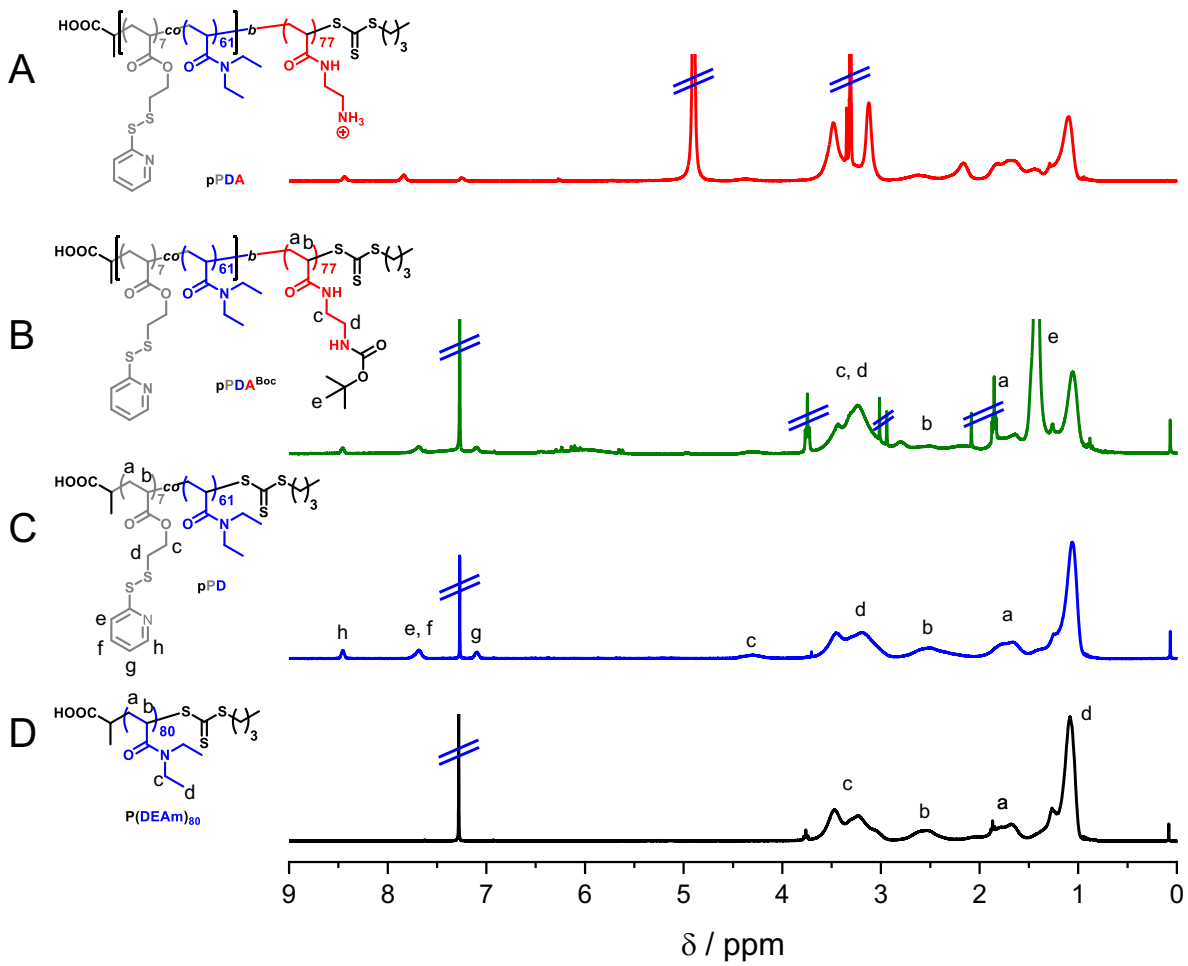

Fig. S3. ^{1}H NMR spectra of pPDA (**A**), pPDA^{Boc} (**B**), pPD (**C**) and P(DEAm)$_{80}$ (pD) (**D**) in $CDCl_3$ and CD_3OD (**A**).

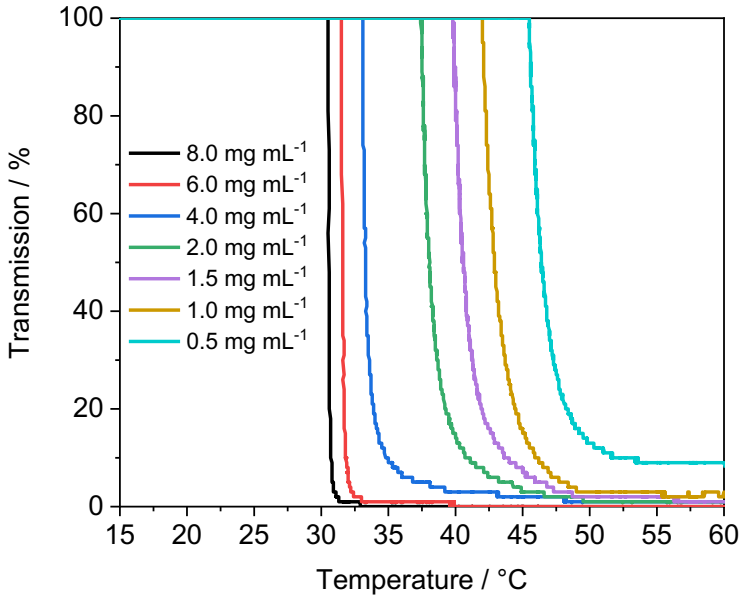

Fig. S4. Graph of the transmission *vs.* temperature of P(DEAm)$_{80}$ in UltraPure water.

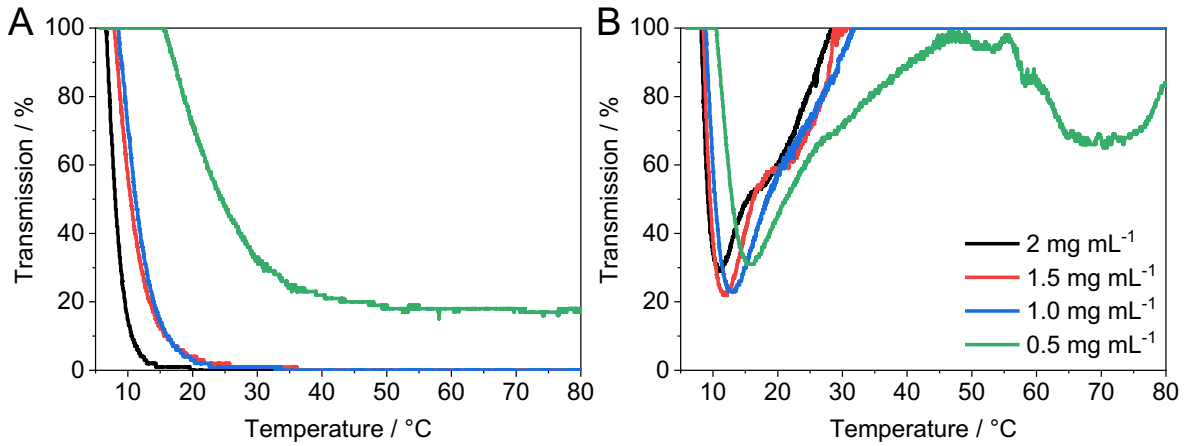

Fig. S5. Graph of the transmission *vs.* temperature of pPDA in (**A**) UltraPure water and (**B**) 150 mM NaCl solution.

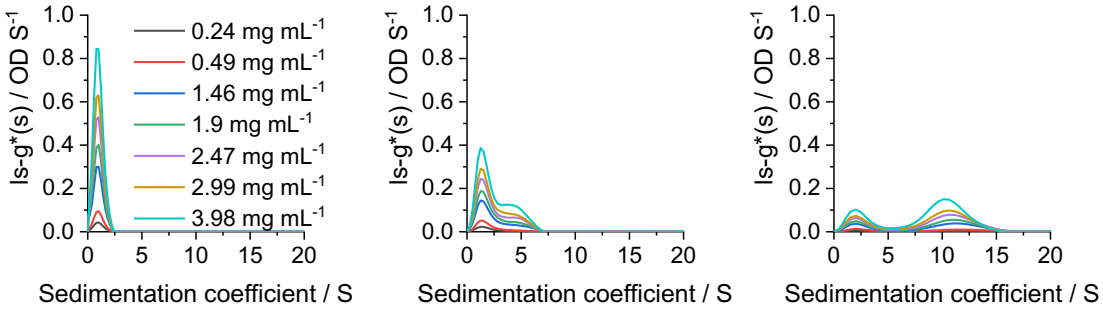

Fig. S6. Differential distributions of sedimentation coefficients, ls-g*(*s*), of pPDA at different concentrations obtained by absorbance detection in terms of OD at 309 nm measured at 5 °C (left), 20 °C (middle) and 40 °C (right).

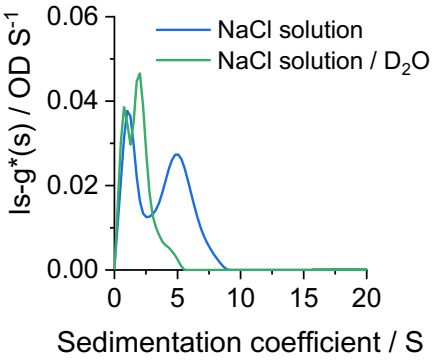

Fig. S7. Differential distributions of sedimentation coefficients, ls-g*(*s*), measured at 20 °C of cross-pPDA^{excess} (1.5 equiv. dithiol crosslinker) in 150 mM aqueous NaCl solution (c = 0.50 mg mL^{-1}) or a mixture of 150 mM aqueous NaCl solution / D_2O (c = 0.47 mg mL^{-1}) for solvent density variation experiments to obtain the partial specific volume (υ = 0.81 cm^3 g^{-1}). Density and viscosity results can be found in **Table S1.**

Table S1. Z-average value ($d_{h,z}$) and PDI of pPDA (1 mg mL^{-1}) in 150 mM aqueous NaCl solution at different temperatures obtained from DLS measurements.

The polymer sample was incubated for 5 min prior to each DLS measurement.

Temperature / °C	$d_{h,z}$ / nm	PDI
25	23	0.480
30	29	0.505
35	29	0.381
40	29	0.268
45	32	0.280
50	33	0.226

Table S2. Results on density and viscosity measurement at 20 °C of the solvent/D_2O mixture for calculations of the partial specific volume (υ = 0.81 cm^3 g^{-1}) as shown in Fig. S7.

	ρ / g cm^{-3}	η / mPa s
NaCl solution / D_2O mixture (48 wt% / 52 wt%)	1.0528	1.1295

Table S3. Results on density and viscosity measurements of 150 mM aqueous NaCl solution at different temperatures for calculations of intrinsic sedimentation coefficients.

T / °C	ρ / g cm^{-3}	η / mPa s
5	1.0067	1.5309
20	1.0044	1.0238
40	0.9983	0.6833

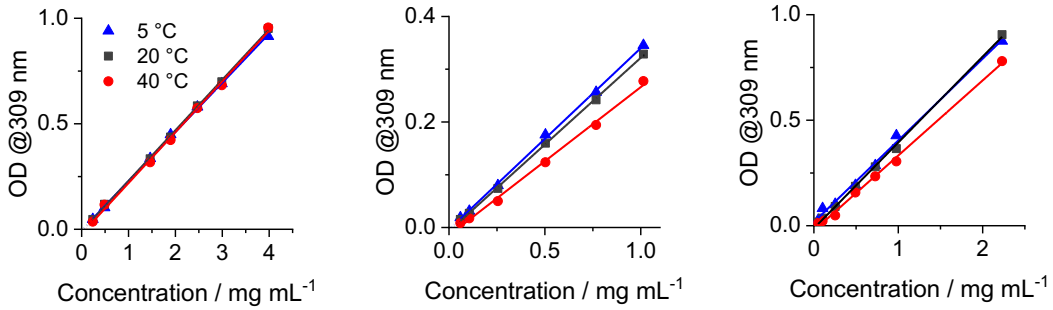

Fig. S8. Linear increase of ODs at 309 nm plotted against sample concentrations at different temperatures of (left) pPDA, (middle) cross-pPDA^{excess}, and (right) cross-pPDA^{dialyzed}.

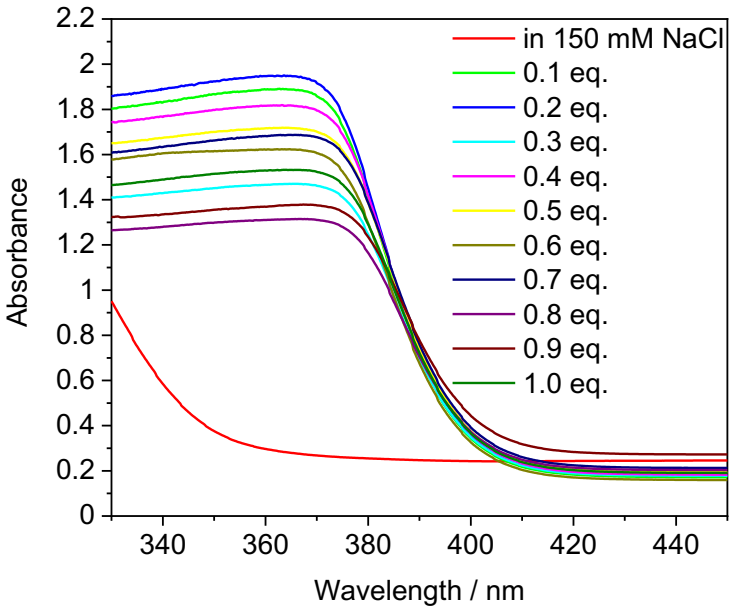

Fig. S9. Graph of the absorbance vs. wavelength of pure pPDA (4 mg mL^{-1}) in 150 mM NaCl and after addition of different amounts of the crosslinker 1,6-hexanedithiol.

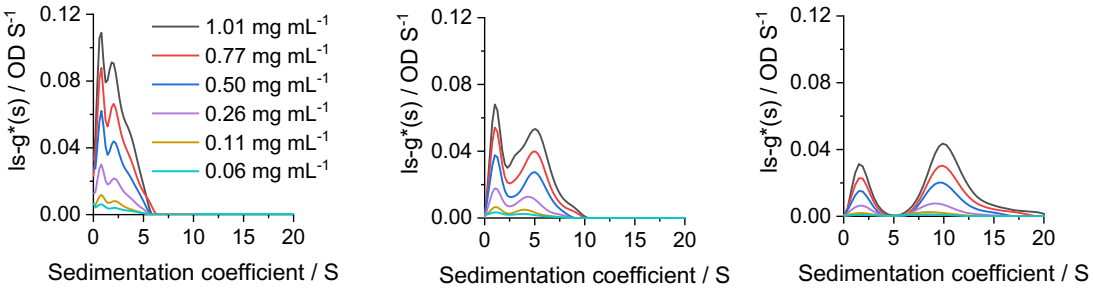

Fig. S10. Differential distributions of sedimentation coefficients, ls-g*(*s*), of cross-pPDA^{excess} at different concentrations obtained by absorbance detection in terms of OD at 309 nm measured at 5 °C (left), 20 °C (middle) and 40 °C (right).

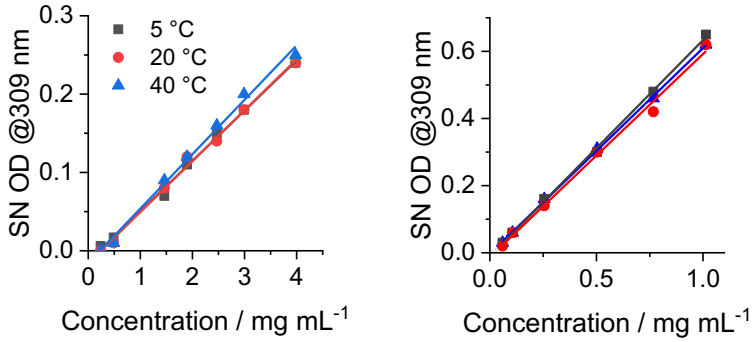

Fig. S11. Increase of ODs at 309 nm of the remaining supernatant (SN) during sedimentation velocity experiments with increased sample concentration measured at different temperatures of (left) pPDA and (right) cross-pPDA^{excess}.

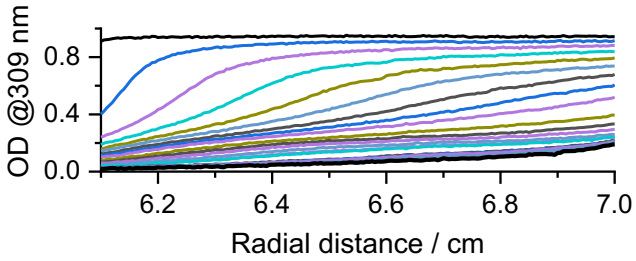

Fig. S12. Radially resolved sedimentation profiles of cross-pPDA^{dialyzed} at 40 °C showing no remaining supernatant at 309 nm after centrifugation.

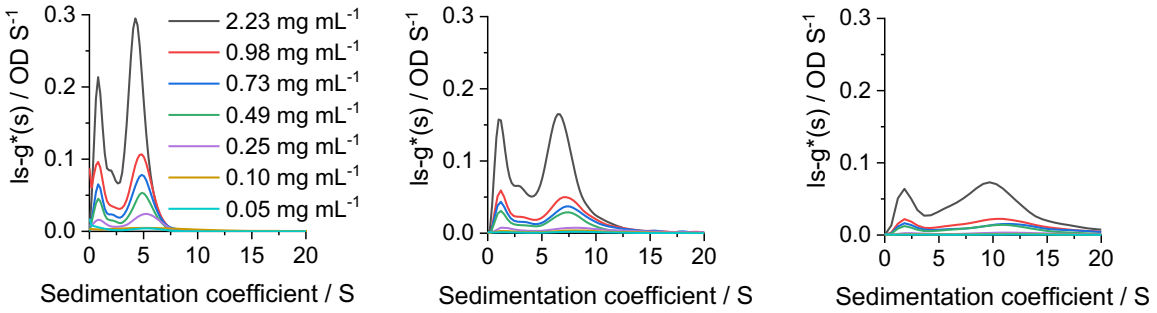

Fig. S13. Differential distributions of sedimentation coefficients, ls-g*(*s*), of cross-pPDA^{dialyzed} at different concentrations obtained by absorbance detection in terms of OD at 309 nm measured at 5 °C (left), 20 °C (middle) and 40 °C (right).

Table S4. Concentration of polymer samples used in different figures in the manuscript.

Sample	Concentration / mg mL^{-1}	Fig.
pPDA	1.00	2, 8, 9
cross-pPDA	4.00	4
cross-pPDA^{dialyzed}	2.23	4
	0.98	6, 7
cross-pPDA^{filtered}	2.17	4, 5, 9, 10
	0.51	8
cross-pPDA^{excess}	1.01	6, 7

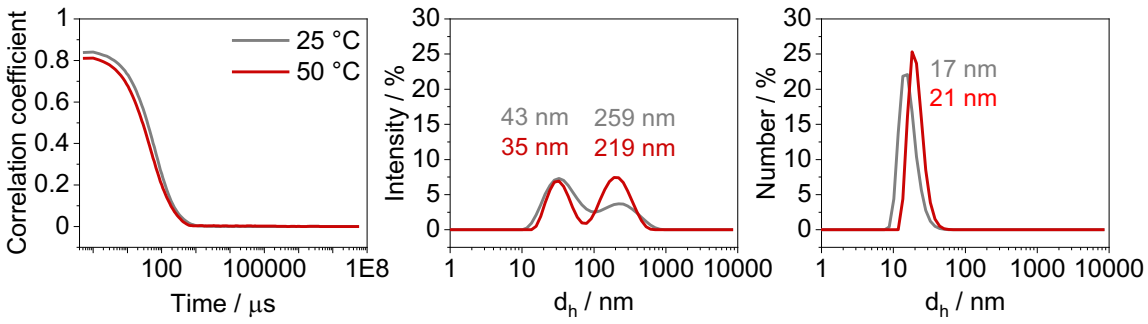

Fig. S14. Exponential decay correlation coefficients and intensity- and number-weighted size distributions of cross-pPDA^{excess} at different temperatures by DLS measurements, the mean size of each population is stated. The polymer solution (4 mg mL^{-1}) was crosslinked with 1,6-hexanedithiol solution (38.9 mg mL^{-1}) in DMSO.

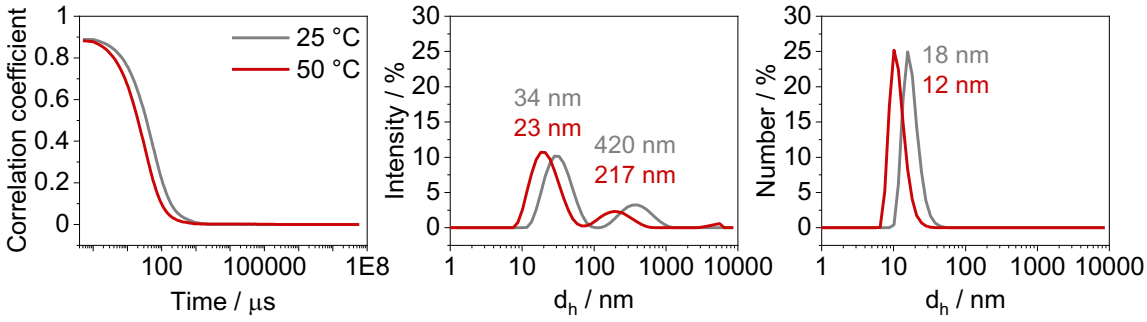

Fig. S15. Exponential decay correlation coefficients and intensity- and number-weighted size distributions of cross-pPDA^{dialyzed} at different temperatures by DLS measurements, the mean size of each population is stated.

Table S5. Z-average value ($d_{h,z}$), PDI, and standard deviation (σ) of pPDA, cross-pPDA, cross-pPDA^{dialyzed} and cross-pPDA^{filtered} in 150 mM NaCl solution at 25 °C and 50 °C obtained from DLS measurements.

The standard deviation was calculated according to eq. S3.

Sample	$d_{h,z}$ / nm		PDI		σ / nm	
	25 °C	50 °C	25 °C	50 °C	25 °C	50 °C
pPDA	23	33	0.480	0.226	16	16
cross-pPDA	41	42	0.379	0.337	25	24
cross-pPDA^{dialyzed}	39	24	0.391	0.350	24	14
cross-pPDA^{filtered}	29	19	0.151	0.126	11	7

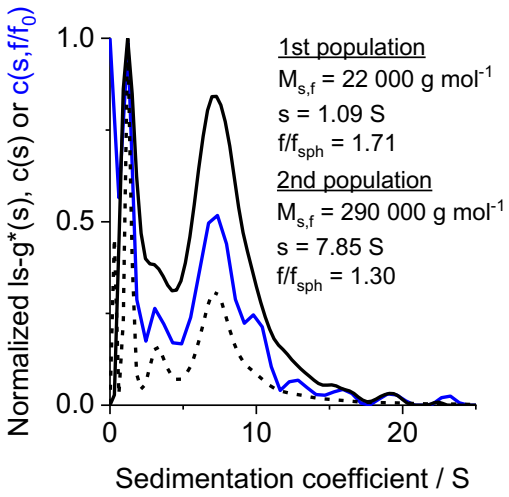

Fig. S16. Normalized differential distributions of sedimentation coefficients, ls-g*(*s*) (black solid line), c(*s*) (black dashed line), c(*s*,*f*/*f*$_0$) (blue solid line) of cross-pPDA^{dialyzed} (at c = 0.98 mg mL^{-1}) measured at 20 °C and evaluated by using the parameters for viscosity and density reported in Tables S1 and S2 and the partial specific volume derived from Fig. S7 (υ = 0.81 cm^3 g^{-1}).

The apparent molar masses ($M_{s,f}$) of the unimer (22000 g mol^{-1}) and the micelles (290000 g mol^{-1}), as well as further results on average sedimentation coefficients, *s*, and frictional ratios, f/f_{sph}, are indicated. For the unimer the integration area was from approx. 0 to 2.4 S and for the micelle from approx. 2.4 to 25 S. An apparent aggregation number of ca. 13 was calculated based on the data and analysis.

Table S6. Size and PDI of polyplexes measured by DLS.

Polyplexes (N/P 10; 30 µg mL^{-1} pKMyc pDNA) were measured by DLS (n = 1).

Polymer/micelle	$d_{h,z}$ / nm	PDI
pA	65.4	0.219
pPDA	85.9	0.185
cross-pPDA^{filtered}	85.1	0.234

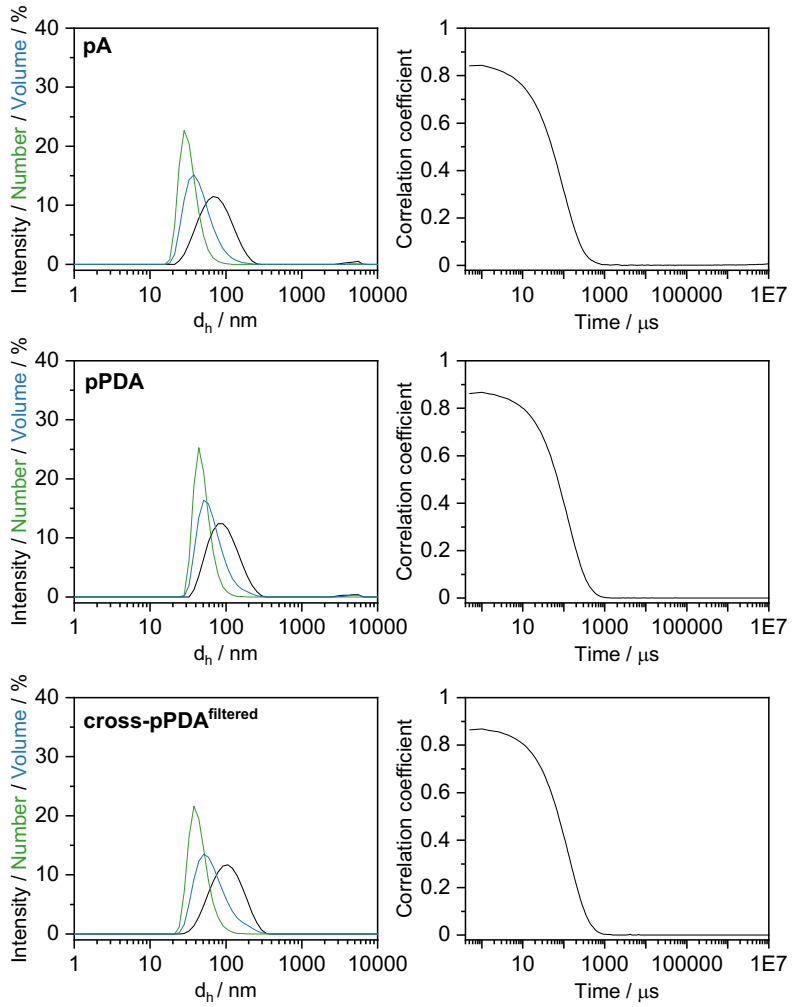

Fig. S17. DLS hydrodynamic diameter distributions and example exponential decays from polyplexes formed at N/P 10 with pA, pPDA and cross-pPDA^{filtered} micelles.

Polyplexes were prepared using 30 µg mL^{-1} pKMyc pDNA. DLS measurements are displayed as exemplary intensity, number and volume weighted distributions and exponential decay correlation coefficients.

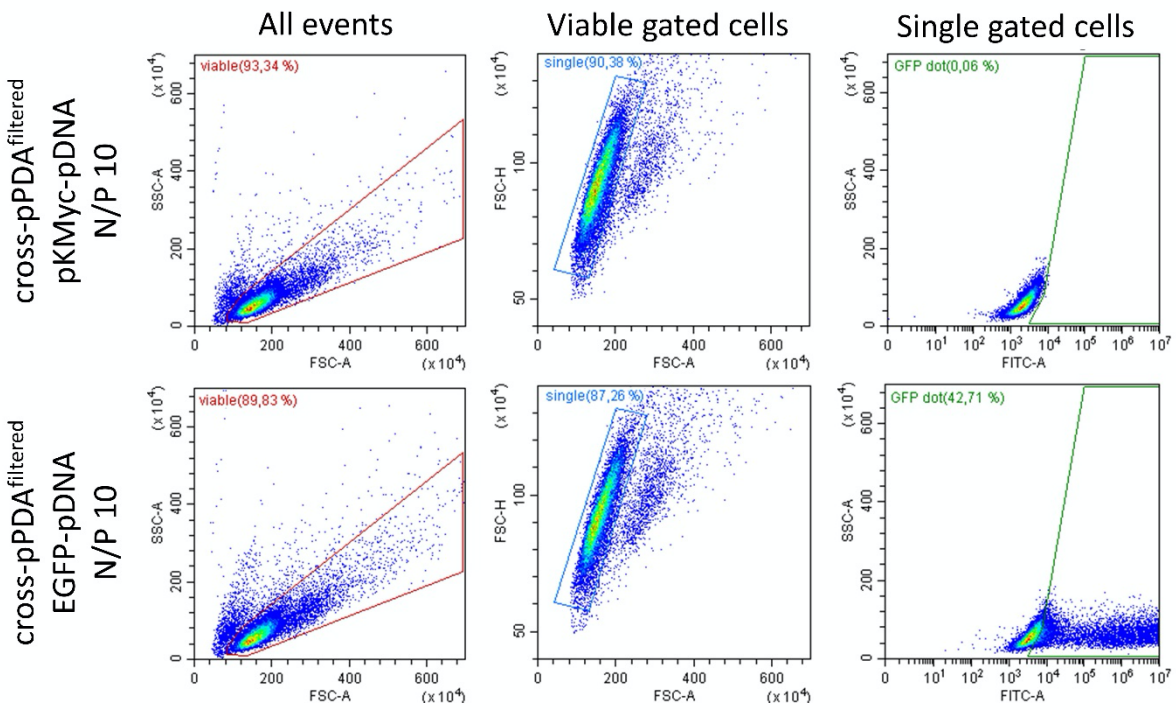

Fig. S18. Gating strategy for transfection experiments.

Viable cells were gated according to the FSC/SSC pattern. Viable gated cells were further gated using the area of FSC signal plotted against the FSC height (FSC-H/FSC-A plot) to discriminate single cells from doublets in the sample. GFP positive cells were identified by gating the single cells to the unstained control (polyplex with pKMyc pDNA).

Table S7. Transfection efficiency of LPEI in HEK293T cells.

LPEI was used as positive control in transfection experiments under optimized conditions (N/P 20, 3 µg mL^{-1} pDNA. Transfection efficiency was measured via flow cytometry and is displayed as percentage of transfected cells in comparison to the control (polyplex with pKMyc pDNA) (n = 3 ± SD).

Polymer	viable fluorescent cells / %
LPEI	82.7 ± 6.3 %

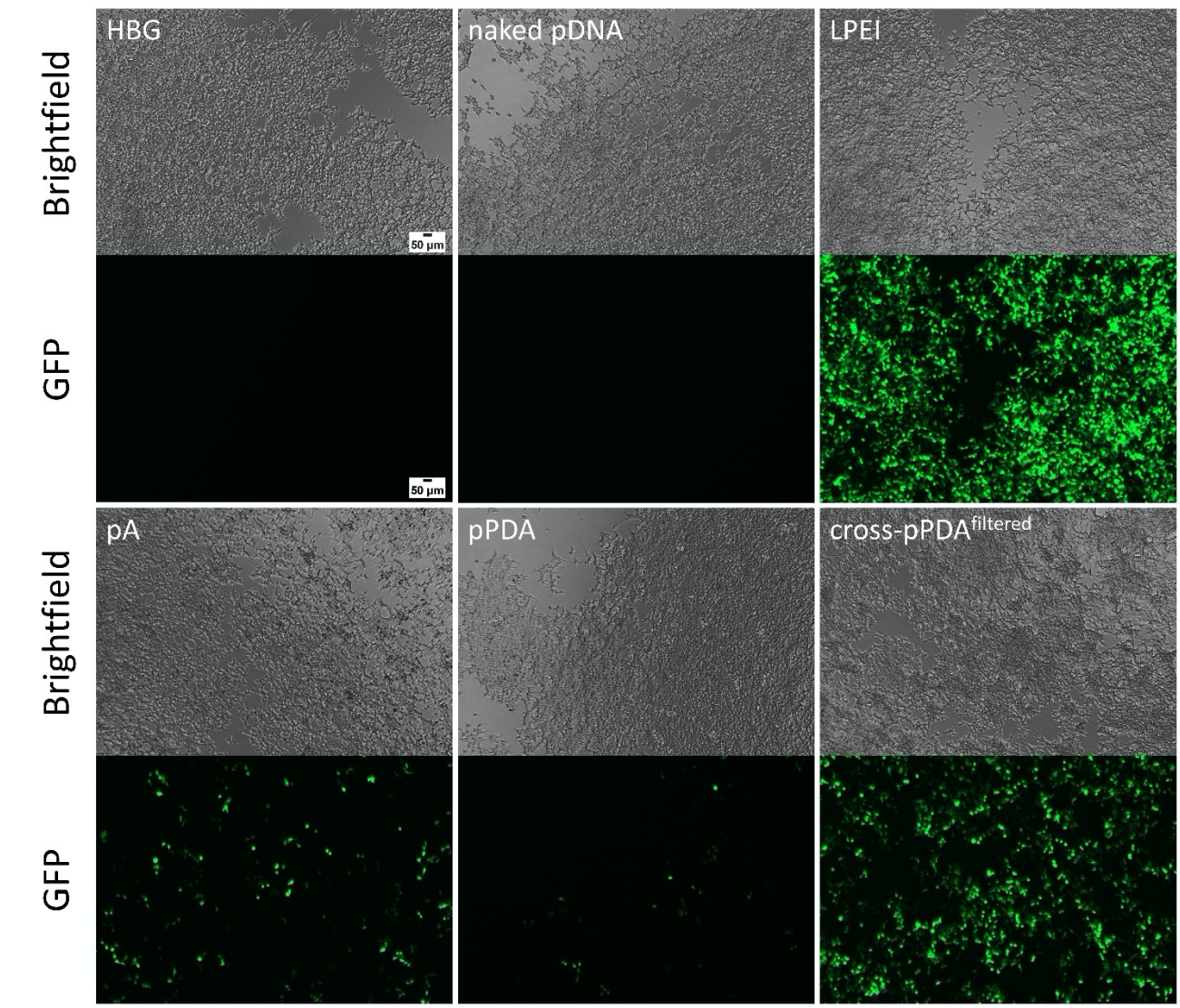

Fig. S19. Brightfield and fluorescence images of HEK293T cells transfected with polyplexes formed with pA, pPDA, and cross-pPDA^{filtered}.

HEK293T cells were incubated with polyplexes for 48 h and imaged *via* fluorescence microscopy. Polyplexes formed with LPEI were used as control.

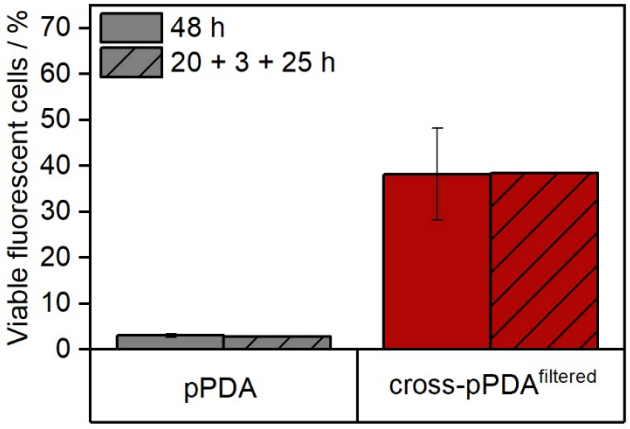

Fig. S20. Influence of temperature change on transfection efficiency of pPDA and cross-pPDA^{filtered} micelles.

The influence of temperature change during cell incubation was evaluated by incubation for 20 h at 37 °C, followed by a 3 h period at 25 °C and subsequent incubation at 37 °C for further 25 h (n = 1). For comparison cells were incubated 48 h at 37 °C. Data for 48 h are already shown in the main manuscript and additionally included here for comparison of the different conditions (Fig. 10, n = 4).

7 References

1. R. Bahadur K. C., B. Thapa and P. Xu, *Mol. Pharm.*, 2012, **9**, 2719-2729.
2. F. Richter, L. Martin, K. Leer, E. Moek, F. Hausig, J. C. Brendel and A. Traeger, *J. Mater. Chem. B*, 2020, **8**, 5026-5041.