# Revisiting the complexation between DNA and polyethylenimine – when and where –S–S– linked PEI is cleaved inside the cell

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1. Synthesis of Linear Polyethylenimine (*l*PEI).

A solution of methyl p-toluenesulfonate (0.186 g, 1 mmol) and 2-ethyl-2-oxazoline (10 mL, 100 mmol) in dry acetonitrile (30 mL) was heated to 90 $^{o}$C and stirred for 48 h under vacuum in a Schlenk flask. The solution was cooled down and precipitated in cold diethyl ether before washed three times by diethyl ether and vacuum dried to result in a yellow powdery poly(2-ethyl-2-oxaline) (PETOZ) with a yield of 80%. $^{1}$H NMR ($CDCl_3$): δ 7.67-7.65 (d, -$C_6\mathbf{H}_4CH_3$), 7.16-7.14 (d, -$C_6\mathbf{H}_4CH_3$), 3.44 (s, -$C\mathbf{H}_2C\mathbf{H}_2$NH-), 2.39-2.29 (m, -$COC\mathbf{H}_2CH_3$ and -$C_6H_4C\mathbf{H}_3$), 1.11 (m, -$COCH_2C\mathbf{H}_3$). GPC (THF as eluent): $M_n$ = 7.6 kg/mol, PDI = 1.12.

PETOZ (5 g) was dissolved into 15% HCl (30 mL) solution. After refluxing for 24 h, white precipitate was filtered and re-dissolved in 30 mL of water. 5 N sodium hydroxide (NaOH) was added into the solution to increase its pH to 9. The precipitate was obtained and washed with water until pH ~ 7. The final product was obtained by freeze-dry (Yield 90%). $^{1}$H NMR ($D_2O$): δ 3.52-3.46 (m, -$C\mathbf{H}_2C\mathbf{H}_2$NH-). $M_n$ = 3.2 kg/mol (as calculated by GPC result of PETOZ).

2. Synthesis of Fmoc-Cys(trt)-Gly-otBu.

Fmoc-Cys(trt)-OH (4 g, 6.83 mmol), H-Gly-otBu (1.26 g, 7.52 mmol), HOBt (0.93 g, 6.83 mmol) and EDC (1.06 g, 6.83 mmol) were dissolved in dry DMF (20 mL). A solution of DIEA (1.19 mL, 6.83 mmol) in DMF (5 mL) was added. The mixture was stirred under the room temperature. After 48 h, the solution was diluted by 50 mL ethyl acetate and washed with 1 N HCl (3 × 100 mL), 5 % $NaHCO_3$ solution (3 × 100 mL) and saturated NaCl (3 × 100 mL). The organic phase was dried over anhydrous $Na_2SO_4$. Then, the removal of solvent by vacuum distillation resulted in a crude yellow foam-like product that was further purified by chromatography using ethyl acetate/hexane (1:1) as eluent. The product was white foam with a yield of 42%. $^1$H NMR ($CDCl_3$): δ 7.53-7.22 (m, -C($C_6$**H**$_5$)$_3$ + Fmoc, 23H), 3.96-3.80 (m, -NH-C**H**$_2$-COO-, 2H), 3.05-3.03 (m, -NH-C**H**($CH_2$-S-C($C_6H_5$)$_3$)-, 1H), 2.82-2.78, 2.60-2.55 (m, -C**H**$_2$-S-C($C_6H_5$)$_3$, 2H), 1.48 (s, -C(C**H**$_3$)$_3$, 9H). ESI-MS: $[M+Na]^+$ = 721.27.

3. Synthesis of Rhodamine-Cys(trt)-Gly-otBu.

Fmoc-Cys(trt)-Gly-otBu (1 g, 1.43 mmol) was dissolved in 20% piperidine/DMF solution (10 mL) and stirred for 2 h to remove the Fmoc group, obtaining $NH_2$-Cys(trt)-Gly-otBu which need not be isolated. The solution was washed with n-hexane (3 × 10 mL). Then, rhodamine B (1.37 g, 2.86 mmol), EDC (0.22 g, 1.43 mmol), HOBt (0.2 g, 1.43 mmol) and DIEA (0.33 mL, 1.43 mmol) were added to the solution. The solution was left to stir at room temperature for 48 h. Then, ethyl acetate (20 mL) was added to the solution. The organic phase was washed with 1 N HCl (3 × 100 mL), 5% $NaHCO_3$ solution (3 × 100 mL) and saturated NaCl (3 × 100 mL) and dried over anhydrous

$Na_2SO_4$. The removal of the solvent in vacuo led to a crude red foam-like product that was further dissolved in $CHCl_3$ (10 mL) and purified by chromatography using a mixture of methanol and dichloromethane (1:9) as eluent. The product was collected as red foam with a yield of 53%. $^1$H NMR ($CDCl_3$): δ 7.95-7.93, 7.48-7.45, 7.09-7.07, 6.52-6.38, 6.15-6.12 (m, rhodamine B, 10H), 7.22-7.16 (m, -C($C_6$**H**$_5$)$_3$, 15H), 3.64-3.53 (m, -NH-C**H**$_2$-COO- + -NH-C**H**($CH_2$-S-C($C_6H_5$)$_3$)-, 3H), 3.35-3.27 (m, -C**H**$_2$$CH_3$, 8H), 2.69-2.62 (m, -C**H**$_2$-S-C($C_6H_5$)$_3$, 2H), 1.43 (s, -C(C**H**$_3$)$_3$, 9H), 1.18-1.12 (m, -$CH_2$C**H**$_3$, 12H). ESI-MS: $[M+Na]^+$ = 925.43.

4. Synthesis of Rho-Cys-Gly-thiolactone.

0.4 g Rhodamine-Cys(trt)-Gly-otBu was dissolved in 4.7 mL of trifluoroacetic acid (TFA). 0.05 mL $H_2O$, 0.125 mL TIS and 0.125 mL EDT were immediately added to the reaction mixture. After 2 h, the solution was diluted with ethyl acetate (40 mL), and washed with 5% $NaHCO_3$ (50 mL), 1 N HCl (50 mL) and saturated NaCl (50 mL). The organic phase was dried over anhydrous $Na_2SO_4$. The removal of the solvent in vacuo led to the product of Rho-Cys-Gly-OH with a yield of 88%. $^1$H NMR ($CD_3OD$): δ 7.92-7.90, 7.60-7.57, 7.14-7.12, 6.52-6.38 (m, rhodamine B, 10H), 3.55-3.48 (m, -NH-C**H**$_2$-COO- + -NH-C**H**($CH_2$-SH)-, 3H), 3.45-3.34 (m, -C**H**$_2$$CH_3$, 8H), 3.14-3.09, 2.92-2.87 (m, -C**H**$_2$-SH, 2H), 1.23-1.16 (m, -$CH_2$C**H**$_3$, 12H). ESI-MS: $[M-H]^+$ = 603.26.

To an ice-cooled solution of Rho-Cys-Gly-OH (0.2 g, 0.33 mmol, in 2 mL of pyridine under $N_2$), acetic anhydride (0.93 mL, 10 mmol, in 1 mL pyridine) was added dropwise over 30 min. The solution was warmed to the room temperature and

stirred for 24 h. The mixture was diluted with 100 mL of $CHCl_3$, washed with 0.1 N HCl (3 × 100 mL), and dried over anhydrous $Na_2SO_4$. After filtration, the solution was concentrated under reduced pressure and precipitated in 50 mL of petrolatum ether. The crude product was rinsed with diethyl ether and then dried to obtain the final product with a yield of 40%. $^1$H NMR ($CD_3OD$): δ 8.00-7.90, 7.80-7.77, 7.48-7.47, 7.28-7.24, 7.06-6.94 (m, rhodamine B, 10H), 3.83-3.80 (m, -NH-C**H**($CH_2$-S-CO-)-, 1H), 3.73-3.65 (m, -C**H**$_2$$CH_3$, 8H), 3.48-3.46 (m, -NH-C**H**$_2$-CO-, 2H), 2.73-2.65 (m, -C**H**$_2$-S-CO-, 2H), 1.34-1.29 (m, -$CH_2$C**H**$_3$, 12H). ESI-MS: $[M+H]^+$ = 587.27.

5. Synthesis of Rho-Cys-Gly-PEI (Rho-PEI).

To a warm solution of *l*PEI (0.54 g, 0.16 mmol, in 30 mL of $CHCl_3$), Rho-Cys-Gly-thiolactone (0.1 g, 0.17 mmol, in 5 mL $CHCl_3$) was slowly dropped over 1 h. The solution was stirred for 24 hrs at the room temperature, concentrated under reduced pressure, and precipitated in diethyl ether. The precipitate was washed by ethyl acetate and acetone and then dissolved in 5 mL water. The solution was flushed through the size exclusion chromatography columns using Sephadex G-50 as a filtration medium. The effluent was collected and lyophilized to obtain the final product with a yield of 80%. $^1$H NMR ($D_2O$): δ 7.80-7.63, 7.30-7.23 (m, rhodamine B), 3.61-3.44 (m, -C**H**$_2$-C**H**$_2$-NH- + -C**H**$_2$$CH_3$ + -NH-C**H**$_2$-COO- + -NH-C**H**($CH_2$-SH)-), 2.59-2.53 (m, -C**H**$_2$-SH), 1.28-1.13 (m, -$CH_2$C**H**$_3$).

6. Synthesis of (Rho-Cys-Gly-PEI)$_2$ ((Rho-PEI)$_2$).

Rho-PEI (0.2 g) and triethylamine (0.3 mL) were dissolved into a mixture of

DMSO and methanol (1:1 v/v, 3 mL). The solution was stirred at the room temperature for two weeks to ensure a maximum reaction and then precipitated in diethyl ether. The crude product was dissolved in deionized water with its pH adjusted to 7 and dialyzed against a large amount of water (2 days) using a dialysis bag with a cut of molar mass ~3 kg/mol (MWCO). The product was freeze-dried (Yield 30%).

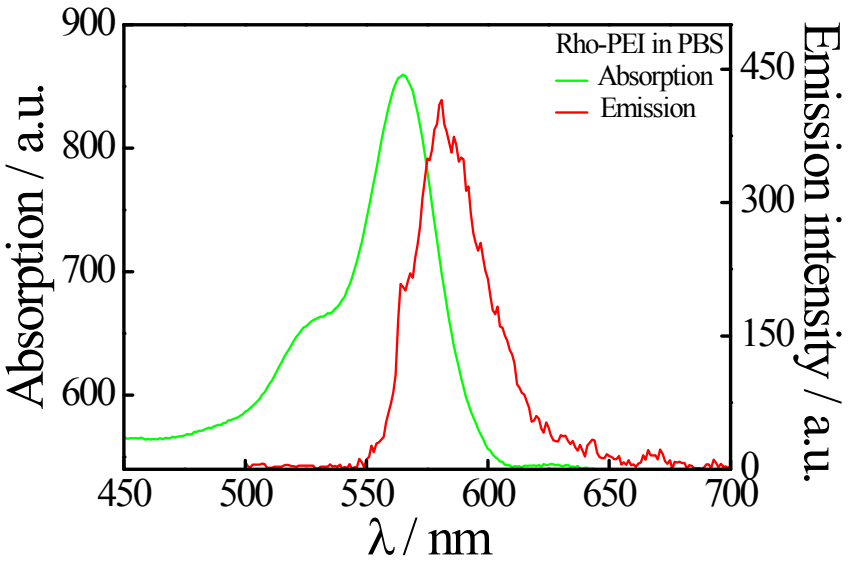


**Fig. S1.** The absorption and emission spectra of Rho-PEI in PBS.

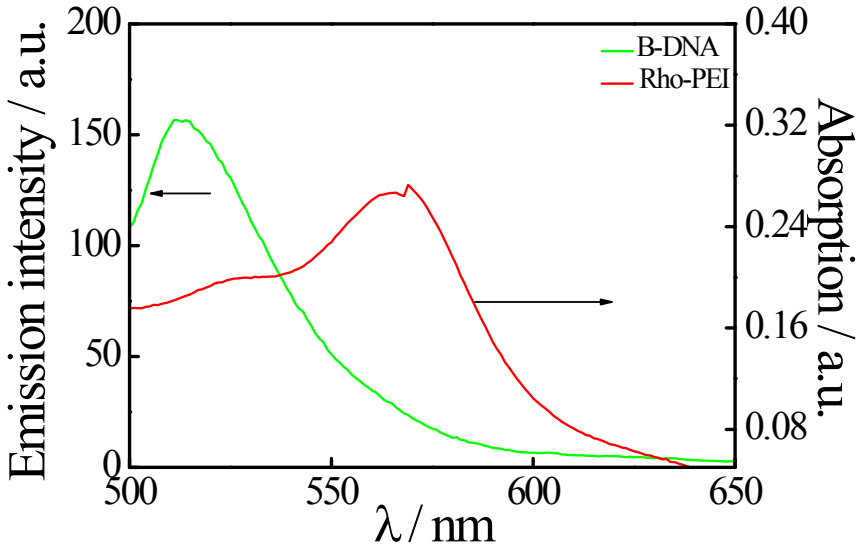

**Fig. S2.** The emission spectra of B-DNA (FRET donor) and the absorption spectra of Rho-PEI (FRET acceptor) in PBS.

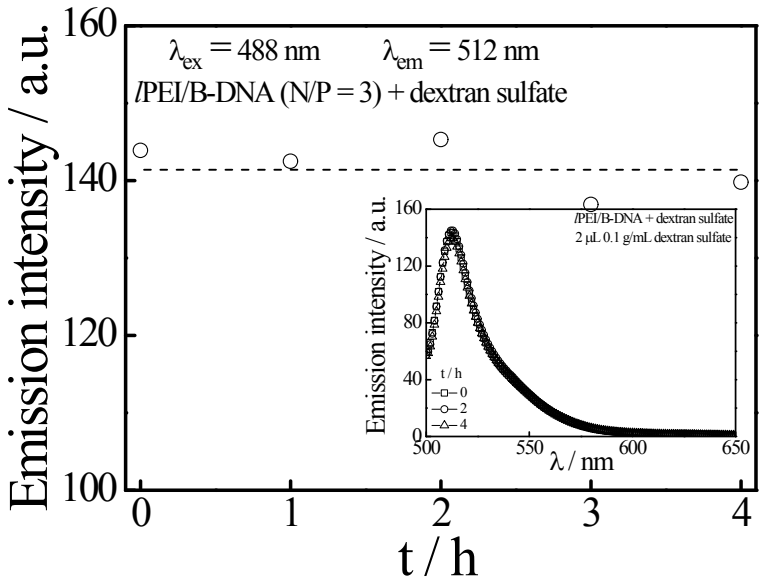


**Fig. S3.** Time dependence of replacement of B-DNA from */*PEI/B-DNA polyplexes (N/P = 3) in PBS by anionic dextran sulfate (0.1 g/mL). The excitation was at 488 nm.

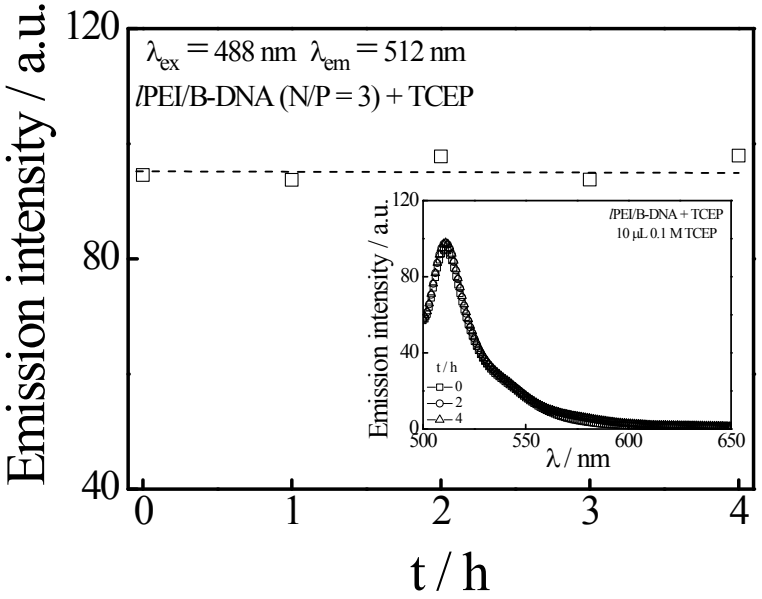


**Fig. S4.** Time dependence of fluorescence intensity of */*PEI/B-DNA polyplexes (N/P = 3) in PBS after treated by tris(2-

carboxyethyl)-phosphine hydrochloride (TCEP). The excitation was at 488 nm.

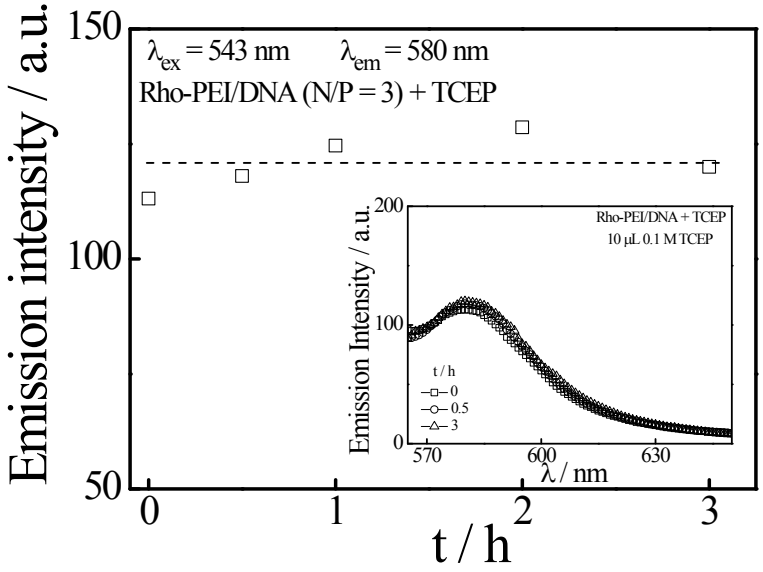


**Fig. S5.** Time dependence of fluorescence intensity of Rho-PEI/DNA polyplexes (N/P = 3) in PBS after treated by TCEP. The excitation was at 543 nm.

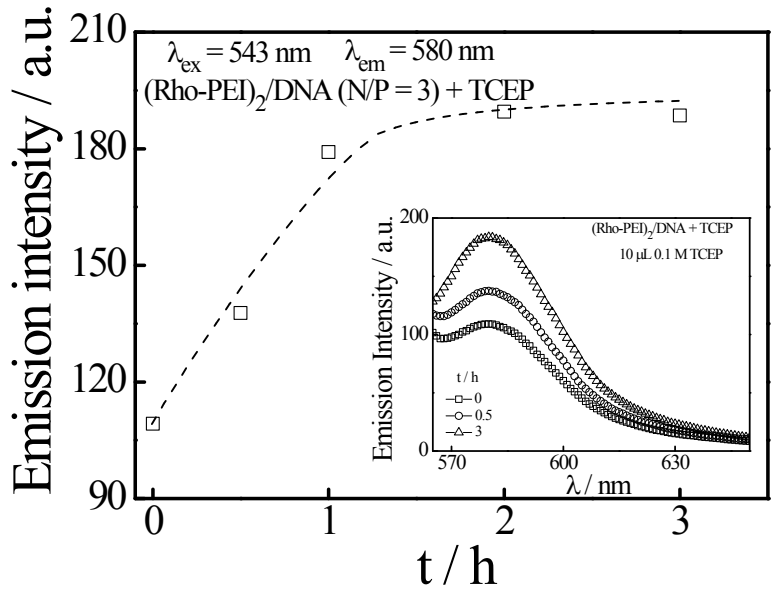


**Fig. S6.** Time dependence of fluorescence intensity of (Rho-PEI)$_2$/DNA polyplexes (N/P = 3) in PBS after treated by TCEP. The excitation was at 543 nm.

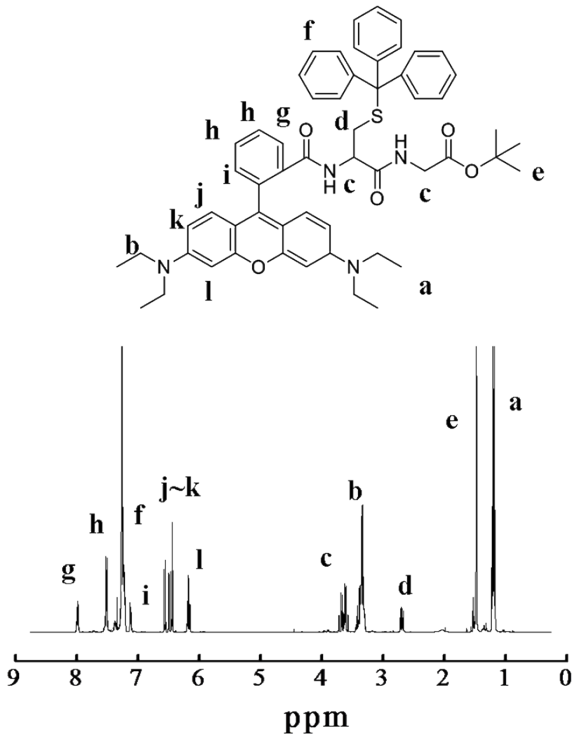


**Fig. S7**. $^{1}$H NMR spectrum for Rho-Cys(trt)-Gly-otBu in $CDCl_3$

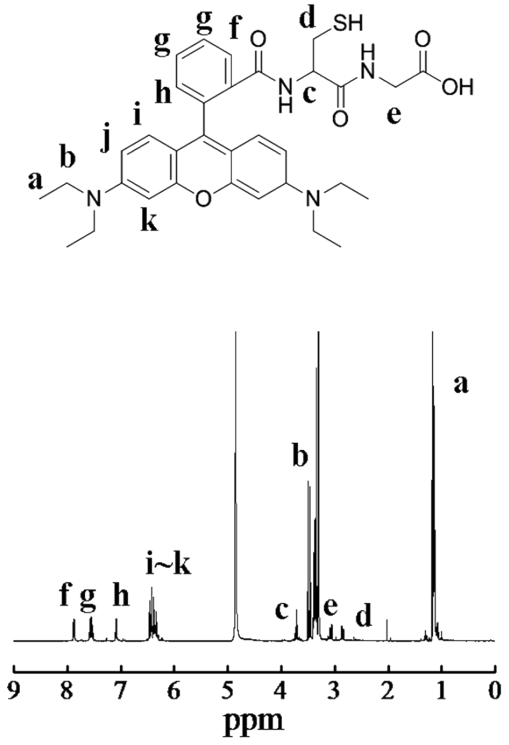


**Fig. S8**. $^{1}$H NMR spectrum for Rho-Cys-Gly-OH in $CD_3OD$

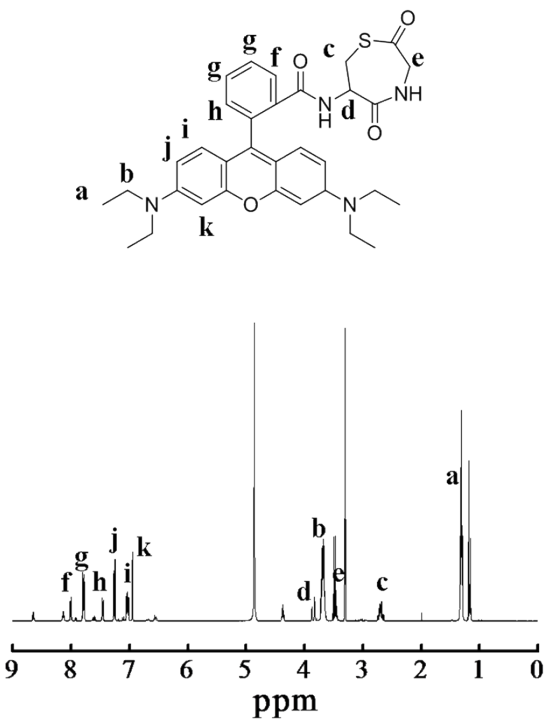


**Fig. S9**. $^{1}H$ NMR spectrum for Rho-Cys-Gly-thiolactone in $CD_3OD$


**Fig. S10**. $^{13}C$ NMR spectrum for Rho-Cys-Gly-thiolactone in $CD_3OD$

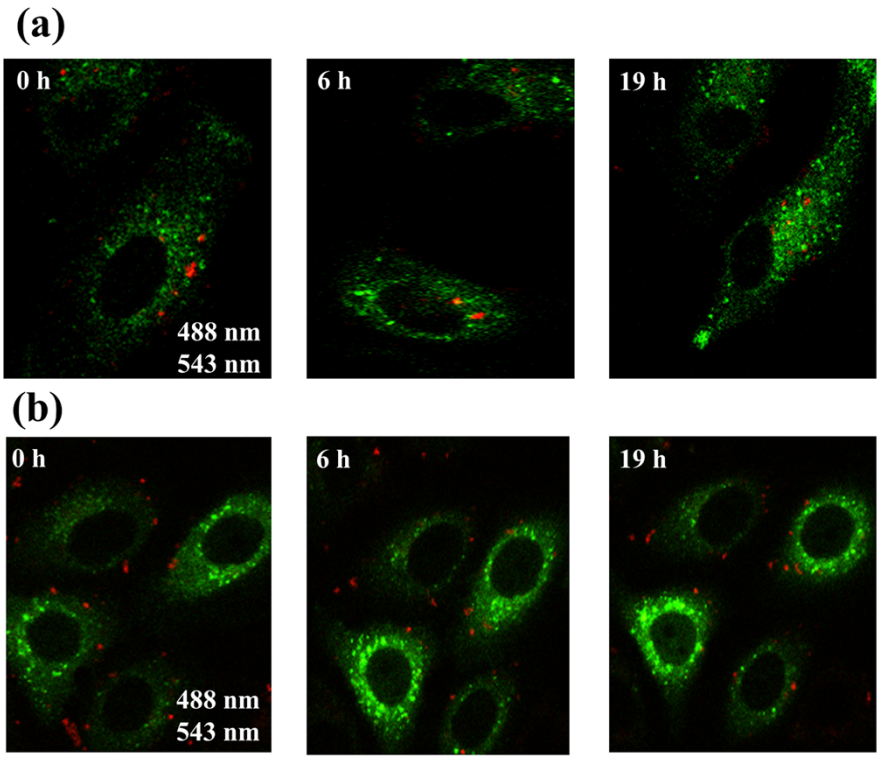


**Fig. S11**. Colocalization of (a) $(Rho\text{-}PEI)_2$/DNA and (b) Rho-PEI/DNA polyplexes with early endosomes labeled by Celllight early endosomes-GFP. Observed at channel 515/30 nm (green) and channel 605/75 nm (red)

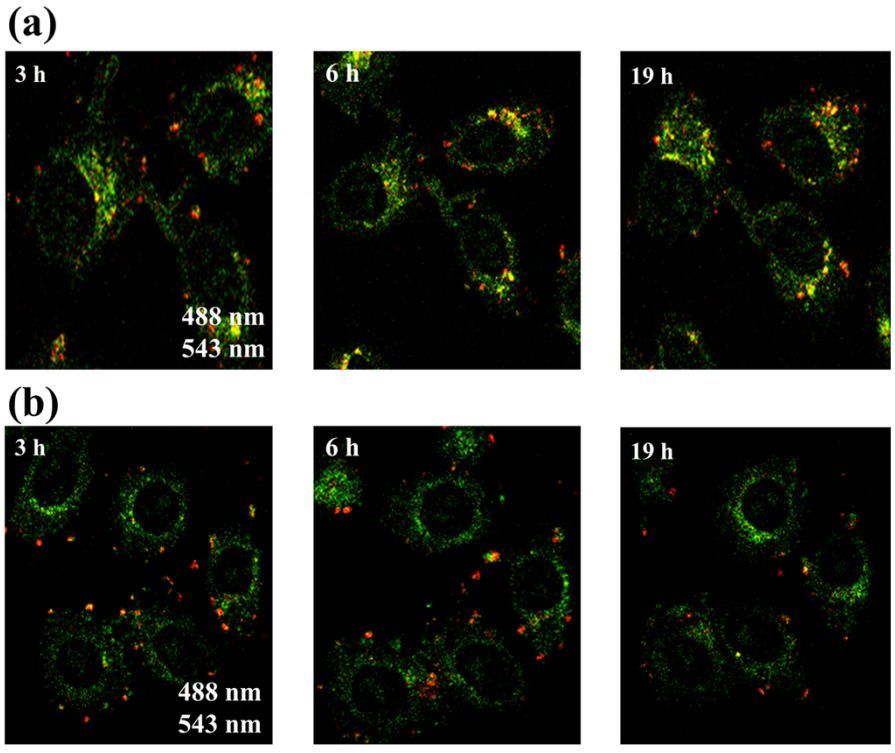


**Fig. S12.** Colocalization of (a) $(Rho\text{-}PEI)_2$/DNA and (b) Rho-PEI/DNA polyplexes

with lysosomes labeled by Lysosensor™ Green. Observed at channel 515/30 nm (green) and channel 605/75 nm (red)