Supplementary Material

The possible "proton sponge" effect of polyethylenimine (PEI) does not include change in lysosomal pH

Rikke V. Benjaminsen[1], Maria A. Mattebjerg[1], Jonas R. Henriksen[1,2], S. Moein Moghimi[3] and Thomas L. Andresen[1,*]

[1]Department of Micro- and Nanotechnology, DTU Nanotech, [2]Department of Chemistry, DTU Chemistry, [1,2]Center for Nanomedicine and Theranostics, Technical University of Denmark, Produktionstorvet 423, 2800 Lyngby, Denmark

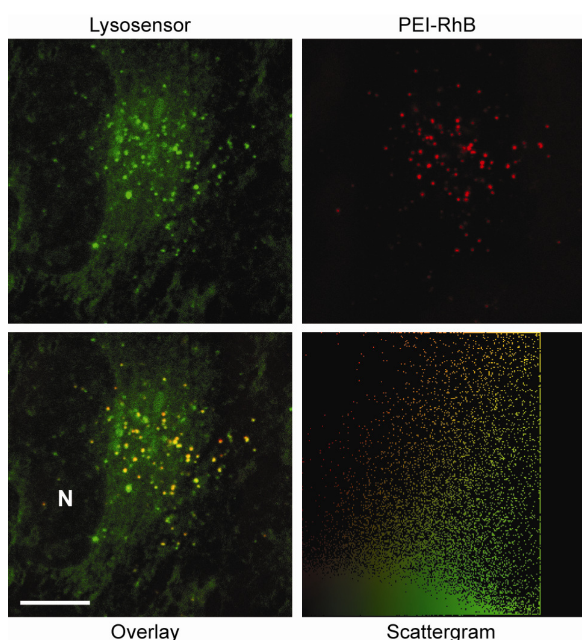
[3]Centre for Pharmaceutical Nanotechnology and Nanotoxicology, Department of Pharmaceutics and Analytical Chemistry, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen Ø, Denmark

*To whom the correspondence should be addressed. Tel: +45 45258168; E-mail: thomas.andresen@nanotech.dtu.dk

Short title: Effect of PEI on lysosomal pH

Colocalisation of PEI-RhB with Lysosensor

2×10^4 HeLa cells per well were seeded in 24-well plates on 9 mm round cover glasses for 24 h. They were then treated with 25 kDa BPEI-RhB at N/P = 7 (corresponding to polyplexes of N/P = 10 with 0.8 µg DNA per well) in full growth medium for 4 h at normal growth conditions. After three hours Lysosensor Green DND-189 was added to a final concentration 1 µM. Cells were then washed three times with ice cold PBS supplemented with heparin (20 unites/mL), once with PBS and kept in imaging medium (DMEM without phenol red and bicarbonate, but supplemented with 30 mM HEPES, 10% FBS and 100 UI/mL penicillin and streptomycin) for observation by confocal microscopy. Supplementary **Fig. S1** shows the colocalization of the green lysosensor with red PEI-RhB. Most PEI has reached a lysosome with lysosensor whereas there are still lysosomes containing only lysosensor.

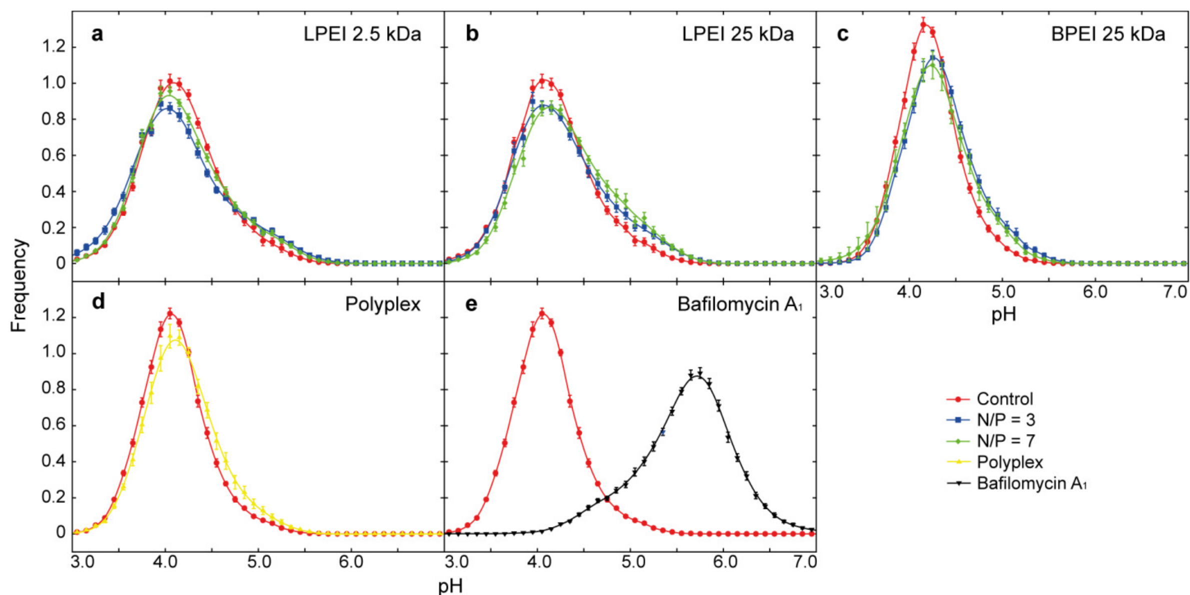


Supplementary Figure S1: Colocalization of RhB-PEI with Lysosensor Green DND-189. HeLa cells were treated with PEI-RhB for four hours and lysosensor for one hour. Top left: lysosensor signal; top right: PEI-RhB; bottom left: overlay and bottom right: scattergram relating the intensity of red to green of corresponding pixels. N: nucleus. Scale bar, 10 µm.

Lysosomal pH measurements in response to different PEI chains and polyplexes.

2×10^4 HeLa cells per well were seeded in 24-well plates on 9 mm cover glasses for 24 h. Cells were then treated with 10 µg/mL nanosensor in full growth medium for 20 h. Cells were then washed three times with ice cold PBS supplemented with heparin (20 unites/mL) and once with PBS. Cells were then kept in imaging medium or treated with PEI, polyplex or bafilomycin A_1. Different free PEI chains were tested: BPEI 25 kDa, LPEI 25 kDa or 2.5 kDa at N/P = 3 and 7 (corresponding to

polyplexes of N/P = 6 and 10 respectively with addition of 0.8 µg DNA per well) as well as a polyplex of BPEI 25 kDa N/P = 6. Treatment was performed in full growth medium for 4 h where after cells were washed with the above mentioned procedure and kept in imaging medium for observation by confocal microscopy. Treatment with 200 nM bafilomycin A_1 was also performed in full growth medium without phenol red for 45 min. For imaging, cells were transferred to imaging medium with 200 nM bafilomycin A_1 without prior washing. Supplementary **Fig. S2** shows frequency distributions of pH measurements of nanosensor containing cells before and after treatment with free PEI, polyplex or bafilomycin A_1. No difference in pH distributions are observed compared to control cells for any of the PEIs and N/P ratios tested. Treatment with complete polyplexes neither revealed any difference in pH distribution. As a positive control is included bafilomycin A_1 treated cells, to verify that an increase in pH can be observed with this method.



Supplementary Figure S2: Measurements of lysosomal pH. Nanosensor internalized during 24 h by HeLa cells imaged by confocal microscopy before (control) and after treatment with LPEI 2.5 kDa, LPEI 25 kDa of BPEI 25 kDa at N/P = 3 and 7 or BPEI 25 kDa polyplex at N/P = 6 or bafilomycin A_1. Histograms show pH distributions of nanosensor allocated signals with mean ± SEM (n = 8 - 12 images). Representative of at least three independent experiments.

PEI content of lysosomes.

HeLa cells were seeded in 24-well plates on 9 mm round cover glasses for 24 h. They were then treated with 25 kDa BPEI-RhB at N/P = 7 (corresponding to polyplexes of N/P = 10 with 0.8 µg DNA per well) in full growth medium for 4 h at normal growth conditions. Cells were then washed washed three times with ice cold PBS supplemented with heparin (20 unites/mL), once with PBS and kept in imaging medium for observation by confocal microscopy. Supplementary **Fig. S3a**

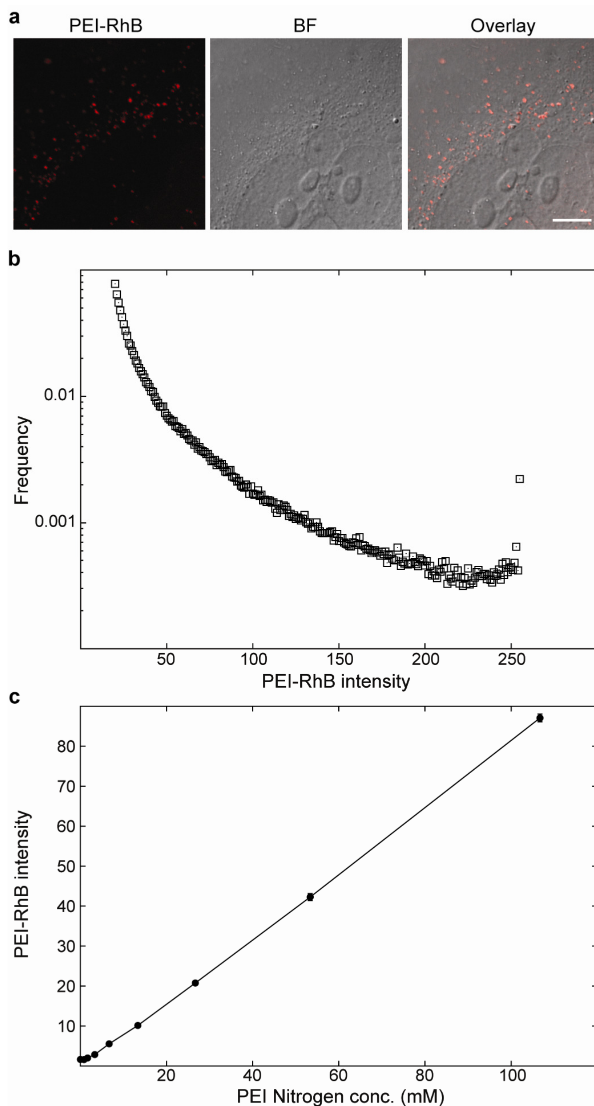
shows the localization of the red BPEI-RhB in cells. **Fig. S3b** illustrates the distribution of the amount of PEI-RhB in cells where the frequency of intensity is plotted. Pixels with an intensity larger than 255 is collected as a point at 256.

In order to determine the concentration of BPEI-RhB in the lysosomes a calibration curve was constructed by diluting free BPEI-RhB in water at descending concentrations of which the mean intensity was measured at the microscope. **Fig. S3c** shows the intensity plotted as a function of PEI nitrogen concentration which yields a straight line:

$$I = 0.81 \cdot c(PEI) + 0.28$$

Where I is the intensity and c(PEI) the concentration of PEI nitrogen atoms, $R^2 = 0.999$.

The frequency distribution of intensities in **Fig. S3b** was converted to a frequency distribution of PEI nitrogen concentrations via the calibration curve in **Fig. S3c**, and presented in **Fig. 6c** of the main article along with the accumulated frequency dristibution.



Supplementary Figure S3: Lysosomal PEI content. a, HeLa cells were treated with BPEI-RhB for 4 hours, washed and imaged by confocal microscopy. Scale bar, 10 µm. **b**, Frequency distribution of intensities of PEI-RhB image in **a**. Pixels with an intensity larger than 255 is collected as a point at 256. **c**, Free PEI-RhB was diluted in water and images were collected at the confocal microscope. Mean intensity ± SD is presented, SD is 1-4%. Representative of three independent experiments.

Supplementary Method: Osmotic pressure and critical size of lysosomes

For every H+ entering the lysosome during acidification one Cl- will enter as well. From the change in concentration of Cl- in the lysosomes the change in osmotic pressure can be calculated:

$$\Delta p = RT\Delta c(Cl^-)$$

Where Δp is the change in ocmotic pressure, *R* is the gas constant, *T* is temperature in Kelvin and $\Delta c(Cl^-)$ is the change in concentration of Cl^-. During acidification of a PEI containing vesicle the pH decreases from pH 7.4 at uptake to pH 4.2 (few lysosomes reach as low as pH 3.2) in the lysosomes and from the titration curve of BPEI 25 kDa it can be determined how much H^+ is needed to decrease the pH from 7.4 to 4.2 per mol of PEI nitrogen atoms. 20 mL of 232 mM PEI nitrogen atoms was titrated with 0.5 M NaOH and 1.9 mL was added to increase the pH from 7.4 to 4.2. This yields:

$$\frac{\Delta n(H^+)}{n(N_{PEI})} = \frac{\Delta n(Cl^-)}{n(N_{PEI})} = \frac{1.9mL \cdot 0.5M}{20mL \cdot 232mM} = 0.2$$

For lysosomes with pH 3.2 this ratio yields 0.26.
$\Delta c(Cl^-)$ can then be expressed as a function of the concentration of PEI nitrogen atoms ($c(N_{PEI})$):

$$\Delta c(Cl^-) = 0.2 \cdot c(N_{PEI})$$

From the Yong-Laplace equation the following relationship between the concentration of PEI nitrogen atoms and the critical size of the lysosomes at which they bursts can be derived:

$$\Delta p = \frac{2 \cdot \tau^*}{r_v^*} = RT \cdot 0.2 \cdot c(N_{PEI})$$

Where τ* is the critical membrane tension where the membrane bursts and r_v^* is the critical radius of the vesicle. The cholesterol content of artificial membranes have been shown to be the main regulator of τ*and the correlation between the two have been shown to be valid for erythrocytes.[1,2] For membranes with a cholesterol content of approximately 20% the correlation yields τ*~10 mJ/m^2. With this critical membrane tension the critical sizes of the lysosomes can be determined from the concentration of PEI nitrogen measured in **Fig. 6c** of the main article and **Fig. S3**:

$c(N_{PEI})$	Critical diameter of lysosome at pH 4.2	Critical diameter of lysosome at pH 3.2	% lysosomes with a conc. > $c(N_{PEI})$
300 mM	260 nm	200 nm	1%
100 mM	775 nm	600 nm	15%
50 mM	1.6 µm	1.2 µm	37%

From these measurements the correlation between $c(N_{PEI})$ and the size of the lysosomes cannot be determined. However, the majority of the lysosomes can become larger than 1.6 nm in diameter before they burst and it is therefore reasonable to conclude that it will only be a fraction, if any, of the lysosomes that will burst because of the “proton sponge” effect. However, it is also known that

it is only a fraction of the internalized polyplexes that actually reach the nucleus and become available for transcription.

References

1. Needham, D, Nunn, R (1990). Elastic-Deformation and Failure of Lipid Bilayer-Membranes Containing Cholesterol. *Biophys J* **58**: 997-1009.

2. Koslov, M, Markin, V (1984). A Theory of Osmotic Lysis of Lipid Vesicles. *J Theor Biol* **109**: 17-39.