Supplementary Information

A proton/macromolecule-sensing approach distinguishes changes in biological membrane permeability during polymer/lipid-based nucleic acid delivery

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Materials and Methods

Materials

Lipofectin Transfection Reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). *In vivo* jetPEI was provided by Polyplus Transfection (Illkirch-Graffenstaden, France). Human Caucasian hepatocyte carcinoma (HepG2) cells were supplied by DS Pharma Biomedical Japan (Osaka, Japan). All other chemicals were obtained from Tokyo Chemical Industry (Tokyo, Japan). Open gate *n*-channel depletion type ISFETs with 40 nm thick Ta_2O_5, 140 nm thick Si_3N_4, and 125 nm thick SiO_2 layers as gate insulator were obtained from Isfetcom co. Ltd. (Saitama, Japan).

Cell culture

HepG2 culture was maintained on 75 cm^2 polystyrene cell culture dishes in Dulbecco's modified eagle's medium (high glucose) with 10% FBS and 1% penicillin/streptomycin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) at 37 °C and 5% CO_2.

Fluorescent labeling of nucleic acids

GL3 luciferase siRNA (Hokkaido System Science, Sapporo, Japan) and firefly luciferase plasmid DNA (pGL4.13, Promega corporation, Madison, WI, USA) were labeled with Cy5 and purified using a Label IT Nucleic acid labeling kit (Mirus Bio, Madison, WI, USA) following the manufacturer's instructions. Labeling and recovery were confirmed using fluorospectrometry and spectrophotometry, respectively.

ISFET-based proton leakage assay

ISFET chips were coated by poly(l-lysine) for cell culture as described earlier[1]. HepG2 cells were seeded in culture medium at $1x10^5$ cells per chip and incubated overnight at 37 °C and

5% CO_2 to attach. For assays at pH 7.4, BTP buffer (1 mM bis-tris-propane, 140 mM NaCl, 4 mM KCl, and 1 mM $MgCl_2$) containing 10 mM CH_3COONa or 20 mM sucrose was prepared. MES buffer (1 mM 2-(N-morpholino)ethanesulfonic acid, 140 mM NaCl, 4 mM KCl, and 1 mM $MgCl_2$) with 10 mM CH_3COONa or 20 mM sucrose was used for measurements at pH 5.5. These buffers were used to dilute the transfection reagents at 1x and 10x the working concentration prescribed by the manufacturer. These dilutions were chosen because the exact concentrations of commercial reagents are not known; For *In vivo* JetPEI, the manufacturer provides a concentration of 150 mM expressed in terms of nitrogen residues, resulting in a recommended concentration of 24 μM of nitrogen residues. Lipofectin is described by the vendor as being a 1:1 formulation of N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE) in membrane-filtered water. Research using a similar liposome formulation was done with a DNA:liposome weight ratio of 1:2.5, which would imply a recommended concentration of 2.5 mg/l Lipofectin[2]. The solutions were alternately superfused over the cells in 60-second cycles at 37 °C using a fluidic system at a flow rate of 100 μL min^{-1}. The outlet of the microfluidic system was placed ~80 μm above the pH-sensing area of the ISFET for fast (<1 s) exchange of the buffer around the cells. For the readout, the sensor was used as a source-drain follower at 0.5 V drain voltage, 0.5 mA drain current, and no DC bias potential against the Ag/AgCl pellet (Warner Instruments, Hamden, CT) reference electrode as described before[1,3-5].

LDH leakage assay

HepG2 cells were seeded in culture medium at $1x10^4$ cells per well in a 96-well plate and incubated overnight at 37 °C and 5% CO_2. The cells were incubated for 15 min at 37 °C with reagent diluted in BTP or MES buffer for pH 7.4 and pH 5.5 conditions, respectively. Then, LDH leakage was assessed in the cell supernatant using a commercial LDH-Cytotoxic Test

(Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions. UV absorbance (A_{560}) was measured with an Infinite M200 microplate reader (Tecan Corporation, Männendorf, Switzerland). Cells treated with 1 mg/ml TX-100 or with buffer were used as positive and negative controls, respectively. Relative LDH leakage was determined as $(A_{560} - A_{560,n.c.})/(A_{560,p.c.} - A_{560,n.c.})$.

Confocal Microscopy

HepG2 cells were seeded in culture medium at $3x10^4$ cells per well on an 8-well chamber slide (Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight at 37 °C and 5% CO_2. Endosomes were labeled using Cell Light Early endosome-GFP or Late endosome-GFP (Thermo Fisher Scientific, Waltham, MA, USA) and the cells were incubated for 24 h. Complexes were formed with Cy5-labeled nucleic acid and Lipofectin or In vivo Jet-PEI, following the manufacturer's instructions. Complexes were added at 600 ng nucleic acid per well and cells were washed with PBS at several time points. Immediately after washing, cell nuclei were stained for 10 minutes with a 1:100 dilution of Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) in PBS. Cells were washed with PBS again and fixed for 10 minutes with 4% paraformaldehyde (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Fluorescence images (2% laser intensity, Cy5 Ex/Em: 633/697 nm, Hoechst Ex/Em: 405/452 nm, GFP Ex/Em: 488/561 nm, pinhole 0.96 AU, 1.1 µm optical section) were taken using an LSM 780 confocal laser scanning microscope (Karl Zeiss, Oberkochen, Germany). Cellular uptake and Manders colocalization coefficient were quantified using Zen software (Karl Zeiss, Oberkochen, Germany)[6].

Supplementary figures

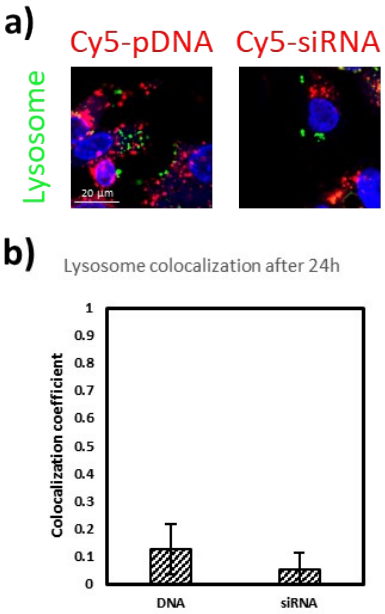

Figure S1. Colocalization of fluorescently labelled pDNA and siRNA with GFP-labelled lysosomes. (a) Representative fluorescence CLSM images of HepG2 cells transfected with Cy5-labelled pDNA or siRNA using *In vivo* JetPEI. Lysosomes were labelled using CellLight GFP. (b) Manders colocalization coefficient of Cy5 with lysosomes determined in 2D-fluorescence images of HepG2 cells with ZEN software. Error bars represent S.D. (n=13 for DNA n=6 for siRNA).

References

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