SUPPORTING INFORMATION PARAGRAPH.

Transition from a Normal to Inverted Cylinder for an Amidine-Bearing Lipid/pDNA Complex and its Excellent Transfection

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S1. Synthesis of TRX

TRX was synthesized according to our disclosed patent (United States Patent 6,228,391, Japanese Patents 2-76810, 2-292246, 4-5226, 4-108391, 4-108391, 6-210155, 6-3229558, 7-316079, 8-27030, 8-59503) and the synthetic scheme is presented in Figure S1. 1H NMR and 13C NMR spectra were recorded on a JEOL JNM-ECP500 spectrometer at 500 MHz and 125 MHz, respectively. In CDCl3, the δH-value is relative to internal Me4Si and the δC-value is referenced to the solvent [δC (CDCl3) 77.0]. MALDI-TOF mass spectra were prepared as micromolar solutions in Methanol; dithranol was utilized as the matrix. All reactions were monitored by TLC on aluminum sheets coated with silica gel 60F254 (0.2 mm thickness, E. Merck, Darmstadt, Germany). Solvent extracts were dried with anhydrous MgSO4 unless otherwise specified.

3, 5-didodecyloxybenzamidine (1).

To a solution of 3,5-dihydorxybenzonitrile (2) and 1-bromododecane (1:3 molar ratio) in acetone was added potassium carbonate, then heated under reflux over night. After water was added, the reaction mixture was extracted with CH2Cl2, washed with saline, dried (MgSO4) and evaporated. The cyano group of the residue (3) was converted to the amidine group by reacting with extra amount of HCl gas
in an dry ethanol/benzene and subsequently with extra amount of NH₃ in CHCl₃. Purification by re-crystallization gave the 1 (yield = 53%). ¹H NMR (CDCl₃) δ in ppm: 0.87-0.89 (t, J = 6.4 Hz, 6 H), 1.26-1.45 (m, 32 H), 1.75-1.79 (quint, J = 6.4 Hz, 4 H), 3.99-4.01 (t, J = 6.4 Hz, 4 H), 6.69 (s, 1 H), 6.86 (s, 2 H). ¹³C NMR (CDCl₃) δ in ppm: 14.1, 22.7, 26.0, 29.1, 29.4, 29.4, 29.6, 29.6, 29.7, 29.7, 31.9, 68.9, 105.3, 107.2, 129.3, 161.2, 166.9. MALDI-TOF MS (M⁺): 489.4411 (Calcd for C₃₁H₅₇N₂O₂, 489.4420).

Figure S1. Synthetic scheme of TRX.

S2. Lipoplex Formulation and Composition

All experiments in this work was carried out at the fixed liposome composition of TRX:DOPE:DLPC = 1:2:1. The chemical structures of DOPE and DLPC as well as diC₁₄-amidine (refs 1&2) are presented in Figure S2. This composition was determined by the optimization so that the transfection efficiency of p-DNA coding β-galactosidase to COS1 showed the maximum. Unless noted, the cation to anion ratio was fixed at 3:1. When we prepared the lipoplex, we used 3 mM PBS buffer. This is because
buffers, especially PBS buffer, disturbed the lipoplex formation. When we increase the PBS buffer concentration above 30 mM, we could not observe complexation between DNA and the liposome.

**Figure S2.** Chemical structures of DOPE, DLPC, and diC14 amidine


S3. **SAXS and Another Evidence to show inclusion of pDNA into the TRX-CL cylinder**

SAXS was measured at 40B2 beam line of SPring8 Japan. The sample to detector distance was 1 m and an imaging plate was used to measure the SAXS intensities. 3 mM PBS was used for the solvent for all experiments.

Figure S3-1 compares the first peaks when TRX-CL was added to pDNA, where the SAXS intensity was normalized by the TRX-CL concentration. The peak area was determined and plotted against the composition in Figure 3-2. With decreasing the TRX-CL composition, the peak area is increased and seems to reach the maximum at TRX-CL:pDNA= 4:6. This feature indicates that the added pDNA was incorporated into the cylinder made from TRX-CL, otherwise the peak area should not depend on the mixing composition.

**Figure S3-1 (left).** Comparison of the first peaks of TRX-CL/pDNA lipoplex. The peak intensities were normalized by the TRX-CL concentrations.

**Figure S3-2 (right).** The normalized peak intensities are plotted against the TRX-CL/pDNA composition.
S4. Estimation of the Size of the CRT-CL/pDNA lipoplex with SAXS

Figure S4 plots $\ln I(q)$ vs. $q^2$ (Guinier plot) for the complexes and pDNA itself and we evaluated $R_g$ (radius of gyration) from the initial slopes indicated in the figure. Since there are some large aggregates, the SAXS curve is up-warded in the smaller q range. Therefore, there is relatively large error in the determination of the initial slope in these plots. Nevertheless, the approximate value of $R_g$ can be determined for each composition. The resultant values can be considered as the size for the major components. It should be noted that the resultant $R_g$ is ranged in 40-100 nm, which approximately agrees with the observed size with TEM.

It should be noted that the observed SAXS intensity is generally expressed by the following equation, here $S(q)$ and $P(q)$ are the lattice and particle scatterings, respectively.

$$I(q) = S(q)P(q)$$

When we evaluated $R_g$, we corrected the contribution from $S(q)$. However, this process may be a reason for the error between SAXS and TEM.
S5. **Comparison of Cytotoxicity between TRX-CL and Lipofectamine2000**

Figure S5 compares the carrier concentration dependence of the proliferation. It is clearly shown that Lipofectamine2000 is more toxic than TRX-CL. Polyamines such as putrescine, spermidine, and spermine are organic polycations present everywhere in cells and can interact with anionic biomolecules. Perhaps because of their ability to bind many biomolecules and their intrinsic toxicity, the transport of these compounds are tightly regulated (Soulet, D., Gagnon, B., Rivest, S., Audette, M., Poulin, R. (2004) J. Biol. Chem. 279, 49355-66. Azzam, T., Eliyahu, H., Shapira, L., Linial, M., Barenholz, Y., Domb, A. J. (2002) J. Med. Chem. 45, 1817-24.) On the other hand, amidine is less popular than polyamines, and thus there should not be no specific.

![Figure S5. Comparison of cell toxicity of TRX-CL and Lipofectamine2000 that depend on carrier concentration.](image)

S6. **Transfection Assay**

Culturing: The hepatoblastoma cell Hep G2 was obtained from the Riken Bioresource center in Japan. Hep G2 cell was maintained in MEM supplemented with 10 % FBS and 1 % sodium pyruvate. The melanoma cell A375 was obtained from the American Type Culture Collection (ATCC). All medium
contained a 1 wt% penicillin/streptomycin mixture and non-essential amino acid contained L-glutamic acid. The cell incubation was always carried out at 37 °C in a fully humidified air containing 5 wt% of CO₂.

Transfection: Hep G2 (1 × 10⁵ cells/ml, 500 µl) were grown just before 24-well plates (Falcon). pEGFP-C1 complexes with TRX-CL or Lipofectamine2000 were added to the medium. After the cells were incubated at 37 °C in 5 wt% CO₂ for 24 hours, the medium was changed to a fresh serum-contained MEM, and the cells were incubated for 48 hours again. The final concentrations of pEGFP, TRX-CL, and Lipofectamine2000 were 4 μg/ml, 105.4 μg/ml, and 105.4 μg/ml. The cells were observed using fluorescence microscopy (IX-70, IX-FLA, Olympus), and there images were captured by AxioVision 3.1 (Carl Zeiss Co., Ltd.).

Flow Cytometry: Hep G2 was grown and transfected according to the procedure described above. Subsequently, the cells were collected by centrifugation in 0.25% trypsin (1,000 rpm., 2 min), and 500 µl of serum-free MEM was added to the collected cells. Fluorescence histogram was obtained determined using an EPICS XL ADC System digital Flow Cytometer (Beckman Coulter). GFP was observed with 516 nm (excited at 498 nm).

Effect of PBS Buffer on the Transfection: Since amidine strongly binds to phosphoric acid, when we mixed TRX-CL and DNA in PBS buffer, precipitation occurred. SAXS showed there was no structural changes occurred and transfection efficiency was not increased as presented in Figure S6.
**Figure S6.** Relation between the transfection and the concentration of PBS buffer. ×10 and ×100 means 10 mM and 100 mM PBS.