A comparative transgene expression study between a protaplex and a rotaplex embedded lipid-nano particles in murine derived dendritic cell

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Abstract
Gene therapy depends on the perfect DNA delivery to the nuclear subdomain, where DNA should be orchestrated in a lipid based programmed packaging, like multi-functional envelope type of nano device (MEND) and tetra lamellar multi-functional envelope type of nano device (T-MEND) published elsewhere. In both the packaging system, DNA has to make a complex (core) with a cationic polymer. It is important how about the effect of such a DNA/condenser core inside the programmed-packaging reflects the transgene expression. Here we compared transgene expression of a firefly luciferase gene from both the protaplex (protamine-DNA) and rotaplex (polyrotaxane-DNA), packaged in MENDs. Dendritic cells were transfected with these nanoparticles and transgene expression, cell viability as well as antigen presentations were measured. Transgene expression from the MEND of protaplex was significantly higher in JAWs-II cell (dendritic cell line) and bone marrow dendritic cell. Cell viability and antigen presentations from the protaplex were also prompt and better than that of polypelex, when T-MEND package systems containing protaplex and rotaplex, were compared. Confocal microscopic studies of fluorescently labeled plasmid DNA reflected the evidence of ease decondensation of DNA from the protaplex than that of a rotaplex. Our results suggest that a natural DNA condenser (protamine in protaplex) prefers transgene expression better to those of synthetic polycationic condenser (polyrotaxane in rotaplex).

Introduction
Gene therapy is advancing now-a-days as molecular medication to treat genetic diseases and metabolic disorders. But it needs a proper programmed packaging device as well as delivery of genetic material to the target site. Through a delivery of nano device, pDNA
should be escaped from the nano device into the cellular nucleus in time. The cargo of nano device should be endocytosed at first, and later, it should be promptly escaped from this compartment before lysosomal degradation. Thereby, cytosol trafficking to the nucleus should be ensured for the better transcription. This is why an ideal gene delivery system should be equipped with a variety of functional devices to overcome these barriers, such as ligands for specific targeting, pH-sensitive fusogenic peptides, and a nuclear localization signal (NLS) (Zabner et al., 1995; Lee and Huang, 1996; Zanta et al., 1996; Kamiya et al., 2003; Kakudo et al., 2004).

With the light of these events, we developed a multifunctional envelop type of nano device (MEND) (Kogure et al., 2004; Kogure et al., 2005) and a modified form of tetra lamellar multi-functional envelop type of nano device (T-MEND) (Akita et al., 2009; Shaheen et al., 2011a; Nakamura et al., 2012). Basically DNA has got the negative charge and it needs to be condensed with positively charged polymer for a prompt packaging. So in both the packaging system (MEND and T-MEND), DNA was condensed to make a complex (core) with a cationic polymer. Previously synthetic polycationic polymers like polyrotaxane and lipofectamine were used to prepare polyplex and lipoplex, respectively (Ooya et al., 2006; Yamashita et al., 2006; Hama et al., 2007; Shaheen et al., 2011b; Kono et al., 2014; Leng et al., 2014; Sakashita et al., 2014). The polyplexes which are formed between cationic polymers and DNA through electrostatic interactions and thus known as polycation/DNA complexes, are by far the most widely used non-viral gene delivery vectors. Many factors such as molecular weight, surface charge, charge density, hydrophilicity and the structure of cationic polymers affect gene transfection efficiency of cationic polymers (Bahadur et al., 2011).

Here we called the polyplex of polyrotaxane as rotaplex. Polyrotaxanes consists of cyclodextrins (CDs) and a linear polymer capped with bulky end groups linked through a biodegradable spacer (Ooya et al., 1995; Ooya and Yui, 1997; Ooya and Yui, 1998; Ooya and Yui, 1999).

Natural cationic polymer like protamine causes condensation of DNA, here, we called it as protaplex, a kind of proteoplex (Chari et al., 2015). The condensed DNA core has a great impact on transgene expression (Yamada et al., 2010). Virtually, DNA needs elongation i.e. it should be decondensed from the polycationic condenser, which is a synthetic polymer in polyplex and lipoplex. Previously, it has been reported that lipoplex revealed three order of magnitude lower transfection efficiency than that of an adenovirus by the post nuclear delivery process (Hama et al., 2007).

Very recently protamine has been choice of condenser of DNA for gene delivery in comparison to other cationic polymers studied in the cancer cell lines (Caracciolo et al., 2011; Pozzi et al., 2014a; Pozzi et al., 2014b). But little information was reported about the condenser effect in immune cell lines like dendritic cell.

As a natural condenser, protamine could be the better choice of safety and avoidance of many unnecessary intra nuclear interactions between polyplex/lipoplex and DNA. Here we reported how a natural condenser reflected better transgene expression from the core of a DNA programmed packaging than that of a synthetic condenser in dendritic cell.

The objectives of using dendritic cells (DCs) are that they are very difficult cells to deliver the gene inside (Nakamura et al., 2006; Shaheen et al., 2011b). They are very professional for antigen presentation in the living body. They are the soldiers of the biological system to recognize and warn against foreign particles (Lewis and Reizis, 2012; Mellman, 2013). DCs play a key role in immunotherapy, providing their ability to process, and present antigens of tumor-derived to T-lymphocytes. They also have capacity to drive the naive T cells to activated tumor-specific effector T-cells. Moreover they are very much familiar to collaborate with natural killer and natural killer T cells as synergistic immunotherapy against tumor as well as B-Cell mediated immunity. Many studies showed that DCs have the potential to act as direct cytotoxic effector cell against tumors, and also as orchestrators of innate-adaptive immune response (Larmonier et al., 2010; Hanke et al., 2013). We compared here, transgene expression of a firefly luciferase gene from both the protaplex and rotaplex DNA, fabricated in lipid nano particles as MEND and we also compared the cell viability as well as antigen presentation of these nano particles.

Materials and Methods

Materials
Dioleoyl phosphatidyl ethanolamine (DOPE) and Cholesteryl hemisuccinate (CHEMS) were obtained from SIGMA-Aldrich CO. (St. Louis, MO). Stearylated octaarginine (STR-R8) and stearylated Lys-Ala-Leu-Ala
(STR-KALA) were custom-synthesized by Kurabo (Osaka, Japan) as described previously (Khalil et al., 2006). Polyrotaxanes (48 DMAE) and Rhodamine labeled polyrotaxanes were gifted by the Professor Yui and his group. Cardiolipin (CL) and phosphatidic acid (PA) were purchased from Calbiochem (Darmstadt, Germany). RPMI 1640 and DMEM were purchased from the local agent of Fisher Scientific Ltd. Plasmid DNA pCMV-luc encoding luciferase was prepared using an Endo Free Plasmid Mega Kit (Qiagen, Germany). QD-545 streptavidin was obtained from Invitrogen. CX-Rhodamine and label IT for biotin kit were purchased from Mirus Bio (Madison, USA).

Animals
The black mice of C57BL/6, which were pathogen free, were purchased from the CLEA JAPAN (Registration number was internal confidential) and the animal related experiment was approved by the Hokkaido university animal care committee.

Methods
Plasmid Construction
Usual pDNA encoding luciferase (GL3) was prepared by inserting a fragment encoding for GL3, obtained by the Hind III/Xba digestion of the pGL3 basic vector (Promega, Madison, WI) into the Hind III/Xba I digested site of pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA) as reported previously (Masuda et al., 2005). The CpG-free pDNA encoding luciferase was constructed using the multiple cloning site of pcPgLfree-mcs (Invivogen, San Diego, CA, USA) as per the protocol published elsewhere (Miura et al., 2015). For the construction of CpG-free pDNA encoding OVA (SIINFEKL epitope), CpG-free insert encoding OVA was custom-synthesized (Invivogen), with CC just above the start codon, and AGCTAGC just below the stop codon to allow these sequences to be cleaved by Nco I (CCATGG), and Nhe I, respectively. The insert encoding CpG-free OVA was obtained by the Nco I/Nhe I digestion, and ligated to the Nco I/Nhe I digested site of pcPgLfree-NEWmcs (pCpGfrees-OVA(0)) (Miura et al., 2015).

Isolation of BMDCs from mice
Bone marrow derived dendritic cells (BMDCs) were generated as per the protocol published elsewhere (Inaba et al., 1992). Briefly, bone marrow cells were cultured overnight in RPMI1640 medium containing 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin, and 10% fetal calf serum (FCS). Non-adherent cells were harvested and cultured in the same medium supplemented with 10 ng/ml GM-CSF. On days 2 and 4, non-adherent cells were removed, and adherent cells were cultured in fresh medium containing 10 ng/ml GM-CSF. On day 6 or 7, non-adhering and loosely adhering cells were used in experiments as mature BMDCs.

Preparation of nanoparticles, MENDs
Multifunctional envelope type of nano device, MENDs were prepared according to the previously published protocols (Kogure et al., 2004; Kogure et al., 2005) (Fig. 1). Before packaging of a MEND, DNA was condensed by protamine with an N/P ratio 2.2 for protaplex, and for rotaplex, DNA was condensed by polyrotaxane (18αCD and 4BDMAE, N/P ratio 5). The N/P ratio between nitrogen to phosphate moiety cannot be used in the same because of charge (zeta potential) and polydispersity index of the condensed core among the DNA, protamine and polyrotaxane. Zeta potential and size are important for the particle formation and also for the cellular uptake of the nanoparticles. Usually the core should be in positive charge and N/P ratio should be optimized to the positive charge. Depending on the number of the amino groups of the protamine and polyrotaxane, the N/P ratio varies. For protamine to DNA, it needs to 2.2 and for polyrotaxanes, it needs to 5.

R8-MEND was prepared as demonstrated previously with some modification (Kogure et al., 2004). First of all a positive core was prepared by condensing pDNA and protamine. The 100 μl of a pDNA solution (0.1 mg/ml in H2O) was added drop-wise to 150 μl of a protamine solution (0.1 mg/ml under vortex (total volume 250 μl). In parallel, a lipid film was prepared by the evaporation of a chloroform solution of 137.5 nmol lipids (DOPE/CHEMS, 9:2) on the bottom of a glass tube. In the case of the KALA-MEND preparation, 5 mol% seaeryl KALA of the total lipid was added i.e. 6.875 nmol of stearyl KALA, which is around 5% of the total lipid in nmol to the lipid mixer was added before addition of the chloroform. The lipid film was hydrated with the pDNA/protamine core particle solution and the hydrated film was sonicated for 1 min in a bath type sonicator (AU-25C; Aiwa Co., Tokyo, Japan) to complete the lipid coating of the condensed DNA to prepare the R8-MEND, 5 mol% (6.875 nmol) stearyl R8 of total lipid was added to the particle solution and allowed to

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stand for 30 min. In the case of the KALA-MEND, 5 mol% of STR-KALA was added gently to the particle solution, prepared from the KALA containing lipid-film and the resulting preparation was incubated for 10 min at room temperature (Fig. 1).

**Preparation of Tetra lamellar MEND, T-MEND**

T-MEND was prepared as demonstrated previously with some modification (Akita et al., 2009; Shaheen et al., 2011a) (Fig. 1). First of all a positive core was prepared by condensing pDNA and protamine. Lipid films composed of DOPE/CL 1/1 (total lipid content: 0.55 mmol) were hydrated with 1 mL of 10 mM HEPES Buffer (pH 7.4) for 10 min at room temperature. The hydrated lipid film was then sonicated using a probe-type sonicator to form small unilamellar vesicle, SUV. Condensed DNA particles were prepared by mixing 50 mL of DNA solution (0.1 mg/mL) with 75 mL of protamine solution (0.1 mg/mL) with vortex (final ratio N/P = 2.2). The suspended nuclear membrane-fusogenic liposomes (inner SUV) and condensed DNA particles were then mixed at a ratio of 2:1 (v/v) to coat the condensed DNA particles with a double-lipid envelope as described previously (Lee and Huang, 1996). A stearylated octa-arginine (STR-R8) solution (20 mol% of total lipid) was added to the suspension of double-layered nuclear membrane-fusogenic particles. Then, this suspension of nuclear membrane-fusogenic particles was mixed with endosome fusogenic liposomes at a ratio of 1:2 (v/v) to generate particles with a double endosome-fusogenic envelope, which we refer to as T-MEND. Stearylated octaarginine (STR-R8) solution (10 mol% of endosome-fusogenic lipid) was added to the suspension of T-MEND to modify the outer envelope with R8. The KALA-T-MEND was prepared as described previously (Hama et al., 2007). A negative protamine-DNA core (N/P, 1) was prepared and two kinds of SUVs of R8 (5%) /KALA (20%) /DOPE/CL and KALA (1.5%)/DOPE/PA were also prepared. First D-MEND (DNA-protamine core fused with the inner SUV named di lamellar MEND, D-MEND) was stabilized by 5 mol % R8 and final T-MEND was positively modified by adding R8 (10 mol%) to make a KALA modified T-MEND (Fig. 1).

The diameter and zeta potential of the MEND were determined using an electrophoretic light-scattering spectrophotometer (Zeta sizer, Malvern Instruments Ltd., Malvern, WR, UK). We used HEPES buffer pH 7.4 for dilution the nanoparticle dispersion to measure zeta potential in Zetasizer Nano machine, using standard operating procedure (SOP) of Malvern soft wire. 50 μl of the nanoparticle suspension and 450 μl HEPES buffer in a disposable cuvette was used to measure the zeta
A low volume quartz batch cuvette was used for size with a small volume of only 20 μl of nanoparticle suspension. The size and electrical potential of all the nanoparticles studied here were mentioned in the Table 1.

**Transfection studies**
For transfection studies, $4 \times 10^5$ cells/well of BMDCs were exposed instantly to 10 μl of the KALA-MEND (equivalent to 0.4 μg plasmid DNA) in serum free medium for 3 h. Thereafter, a medium containing 10% FCS was added, and the resulting suspension was incubated for an additional 21 h. Through the transfection studies, GM-CSF was added in the medium at 10 ng/ml. The cells were then washed and solubilized with the reporter lysis buffer (Promega, Madison, WI). Luciferase activity in the cell lysate was then measured by means of a luminometer (Luminoscencer-PSN; ATTO, Tokyo, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (PIERCE, Rockford, IL).

**Cytotoxicity studies (Tetra Color One) assay**
In the cell viability assay, cells were seeded on 24-well plates at a density of $4 \times 10^4$ cells per well. One day later, the cells were washed once with serum-free DMEM followed by incubation in serum-free DMEM in the presence of MEND and T-MEND particles encapsulating 0.4 μg of pDNA for 3 h. The medium with nanoparticles was then removed and replaced with 1 ml of fresh medium containing 10% FBS. Cell viability was assessed as a function of NADH content using the Tetra Color ONE cell proliferation assay system (Seikagaku Kogyo, Tokyo, Japan), a mixture of two reagents, 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazorium, monosodium salt (WST-8), and 1-methoxy-5-methylphenaziniummethylsulfate (1-methoxy ‘PMS’). After a 3 h incubation of the MEND and T-MEND treated cell, Tetra Color ONE reagent was added to each well in medium containing 10% FBS and further incubated for 1 h, cell viability was then assessed by colorimetry at the wave length of 450 nm using a microplate spectrophotometer (Benchmark Plus, Bio-Rad Laboratories). As MTT, WST-8 in Tetra Color One is a water soluble tetrazolium salt, which can be reduced to a formazan product by active mitochondrial dehydrogenase enzyme in living cells. Contrary to MTT, however, this formazan product is a water soluble, and can be directly measured by a spectrophotometer without any extraction procedure for the living cell assay. Tetra Color One assay requires only a one-step procedure before spectrophotometric measurement and it measures intracellular dehydrogenase activity of the viable cell (Yamamoto et al., 2001). All samples were tested in triplicate.

**Antigen presentation**
DC cells were transfected using T-ME (OVA) antigenic plasmid containing SIINFEKL epitope (Karttunen et al., 1992; Karttunen and Shastri, 1992; Shastri and Gonzalez, 1993), which were packaged with KALA modified MENDs/T-MENDs. After 24 h, the transfected DCs were harvested using trypsinization (0.25% trypsin, 0.03% EDTA), followed by centrifugation at 15000 rpm for 3 min. The transfected BMDCs ($2 \times 10^5$ cells) were mixed with B3Z T-cell hybridoma ($1 \times 10^5$ cells) in RPMI 1640 with 10% FCS in 96-well plates, and incubated for 15 h at 37 °C (47).

B3Z illustrates for the term B3286/90.14, which is a somatic T cell, kind of hybrid cell, originated from the fusion of OVA (SIINFEKL) specific Kβ allele i.e. OVA/Kβ specific CD8+ clone, B3, with a lacZ-inducible origin of BW5147 fusion inserts (Escherichia coli p-galactosidase or LacZ construct). Accordingly, when peptide/MHC-

| Nanoparticles       | Size    | PDI  | Zeta Potential |
|---------------------|---------|------|----------------|
| KALA MEND (Prx)     | 153 ± 10| 0.3  | 42.2 ± 2.5     |
| KALA MEND (Prt)     | 123 ± 8.5| 0.18| 32.2 ± 3       |
| R8 MEND (Prx)       | 176 ± 9 | 0.3  | 35.2 ± 2.5     |
| R8 MEND (Prt)       | 190 ± 22| 0.28 | 43.7 ± 2.6     |
| R8 T-MEND (Prx)     | 210 ± 8.5| 0.18| 39 ± 3.2       |
| R8 T-MEND (Prt)     | 157 ± 18| 0.12 | 42 ± 1.6       |
| R8 KALA T-MEND (Prx)| 196 ± 1.23| 0.2  | 43 ± 4.8       |
| R8 KALA T-MEND (Prt)| 205.6 ± 1.6| 0.2 | 41.8 ± 1.7     |

*Table 1. Dynamic light scattering studies of the nanoparticles, containing a protaplex and that of a rotaplex.*
specific response to B3Z T Cells were occurred, consequent *Escherichia coli* β-galactosidase can be measured, known as lacZ assay (40).

The co-cultured cells were then washed with 200 ml PBS, and then incubated with 100 μl of chlorophenol red β-D-galactopyranoside buffer (5 mM chlorophenol red β-D-galactopyranoside, 0.5% NP-40, and 9 mM MgCl2 in PBS) for 4 h at 37 °C. After the incubation, the absorbance at 595 nm of each well was measured using a micro plate reader (Benchmark Plus; Bio-Rad). Antigen presentation is denoted as the fold increase taking the values for untreated DC/T-cells as one.

FRET optimization for visualization of condensation/ decondensation status of p-DNA in fluorescently labeled rotaplex and protaplex.

FRET was optimized by labeling both plasmid DNA with QD 545 as donor and acceptor cation polyrotaxane with rhodamine to make a fluorescently labeled rotaplex as per the previously published protocol (Akita et al., 2009). The pDNA was labeled according to the manufacturers recommended protocol of a Mirus Label IT® CX-biotin nucleic acid labeling kit. FRET was optimized by partially replacing 48DMAE-ss-PRX with rhodamine-DMAE-ss-PRX. The synthesis of rhodamine-DMAE-ss-PRX was described in the previous publication (Akita et al., 2009). Keeping a charge ratio constant at 5, fluorescence was measured in a real time fluorescence photometer (JASCO, FP-750 spectrofluorometer, Tokyo, Japan), where the QDs were excited at 340 nm and an emission spectrum was collected from 550 nm to 650 nm. FRET had been optimized for excitation so far as possible to lower wavelength in order to get the perfect emission effect (Fig. S2). In figure S3, fluorescent probe was optimized for QD-FRET while in condensed form of DNA-Polyrotaxane.

To visualize protaplex, here a double labeled plasmid DNA was prepared. First the DNA was labeled with CX-Rhodamine as per manufacturer protocol and thereafter the same DNA was labeled with biotin kit (Fig. S1). The fluorescently labeled DNA was precipitated by NaCl-ethanol precipitation method. After drying the DNA, the concentration was measured by a UV spectrophotometer. Adjusting the concentration of DNA as per previous protocol (Akita et al., 2009), QD 545 streptavidine was labeled accordingly and purified by spin column Sephadex G-50. Thereafter fluorescence was checked for the double labeled plasmid DNA accordingly (Fig. S1).

Confocal image studies of condensation/decondensation status of a fluorescently labeled protaplex and rotaplex in live cell.

For confocal image studies 5x10⁴ cells/well were seeded on 35-mm glass base dishes (IWAKI, Tokyo, Japan) for 2 days, and then transfected with T-MEND containing 0.4 μg of QD-labeled DNA condensed with acceptor-labeled DMAE-ss-PRX at 37 °C under an atmosphere with 5% CO2. At 3 h after transfection, medium was changed with fresh culture medium, and then incubated for additional 3 h. Cells were washed by 5 mM HEPES containing 20 U/mL of heparin sodium to remove the T-MEND bound on the cell-surface. Thereafter, medium was replaced with HEPES buffer (135 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES and 10 mM glucose, pH 7.4), and examined by confocal

![Figure 2. Transgene expression in dendritic cell line (JAWS-II):](image-url)

First two bars from the left were for the R8-MEND and the second two bars were for the KALA modified MEND. In both pairs, the nanoparticles consisted the protaplex and rotaplex. (+) CM (R5) (PRX5) means positive DNA-polyrotaxane core (rotaplex) of N/P ratio 5, encapsulated with lipid bilayer of DOPE/CHEMS (CM), which was further modified with stearylated R8 (5%); (+) CM (R5) (Prot 2.2) means positive DNA-protamine core (protaplex) of N/P ratio 2.2, encapsulated with the same lipid bilayer of DOPE/CHEMS. *P < 0.05 and ***P < 0.01 unpaired two tailed student t test, followed by Mann-Whitney test.
laser scanning microscopy (LSM 510 META; Carl Zeiss Co. Ltd. Jena, Germany) equipped with an oil-immersion objective lens (Plan-Apochromat 63x/NA 1.4). The QD was then excited by light (488 nm) from an argon laser. Spectral data are presented as relative fluorescence intensity compared to maximum intensity (545 nm).

**Statistical Analysis**

All the experiments were performed triplicate and data were analyzed by Graph Pad Prism software. **P < 0.03 and ***P < 0.01 unpaired two tailed student t test were performed, which were tested by Mann-Whitney test.

**Results**

In figure 2 we found significantly higher transgene expression of MENDs, containing protamine core (N/P 2.2) inside than that of MENDs, containing polyrotaxanes (PRX-48) core (N/P 5). It has been found in both the dendritic cell line (JAWS-II) and the bone marrow derived dendritic cells (BMDC). In dendritic cell line, this difference was around 2 order of magnitudes of gene expression for R8 MEND (first two bars of left hand side in Fig. 2) and almost one and half order of magnitudes for KALA MEND (last two bars of right handed side in the same Fig. 2). Getting this difference in the dendritic cell line, we generated bone marrow derived dendritic cells (BMDC, usually known as primary cell) later and transfected with the same nano-devices, containing the same plasmid DNA encoded GL3. We found here also one order of magnitude in transgene expression form the nano particles containing two different cores of synthetic and natural condensers, respectively (first two bars of left hand side for rotaplex and last two bars of right handed side for protaplex in Fig. 3). An increased dose of DNA contributed around one order of magnitude in transgene expression. The same effect was found in each of the packaging system. But the difference between the protaplex and rotaplex was the same as before.

We then increased the dose of the DNA and these increased-dose also made around one order of magnitude in transgene expression, when the same dose of plasmid DNA was compared (Fig. 3).

It was significant difference in transgene expression between the nanoparticles containing DNA cores of PRX and protamine core. Thereafter we performed cellular toxicity studies and antigen presentation of the nanoparticles, containing the same cores. In every

![Figure 3. Transgene expression in bone marrow derived dendritic cell (BMDC): The left side bars were the luciferase gene expression from the nanoparticles containing rotaplex and right side bars containing protaplex. BMDC were generated and transfected with the respective MENDs for 24 h and then luciferase gene expression was measured after lysis and luciferin assay. The gene expression from the rotaplex showed dose depended expression and that of from the protaplex showed up to the 0.8 µg per 500 µl media containing the cell 4 x 10^5. There is a significant difference between a rotaplex and protaplex containing luciferase gene in the figure. **P < 0.03 and ***P < 0.01 unpaired two tailed student t test, followed by Mann-Whitney test.](image-url)
case it has been found that the DNA nanoparticles containing protamine cores i.e. protaplex, played the better in antigen presentation as well as cell viability in comparison to those from the nano device, containing rotaplex of PRX(Fig. 4 and Figure 5). In Figure 4 of Tetra One Color assay, the NADH function was 70% and the lower in the case of the nanoparticles, containing rotaplex (Fig. 4a) and that of protaplex showed more than 90% (Fig. 4b). We compared the cell viability of the DNA nanoparticles packaged in both the MEND and T-MENDs. In Figure 4 b, however, an increase of almost 40% in cell viability after only 3 hours from the removal of the treatment is unusual.

Moreover, when the plasmid was changed with that of antigenic plasmid containing SIINFEKL epitope (Karttunen et al., 1992; Karttunen and Shastri, 1992; Shastri and Gonzalez, 1993) and co-cultured with the respective B3Z T-cell hybridoma, better antigen presentation was also found in case of nano particles, containing protaplex. MTT assay showed also the better tolerance in case of the nano particles containing the protaplex rather than rotaplex. QD-FRET in live cell imaging (Fig. 6) showed comparatively larger area of green signals in fluorescently labeled protaplex than that of a rotaplex, which means highly decondensed core favored transcription for gene expression. On the other hand, rotaplex labeled nanoparticles showed larger area of red signal, which means highly condensed core between the DNA and polyrotaxanes. It means very slow transcription for gene expression in synthetic condenser, polyrotaxane.

Figure 4. Cell viability as a function of NADH by Tetra ColorOne assay: a) Cell viability against R8 MEND, R8 TMEND and R20/K5 (%) TMEND, containing a rotaplex. The nanoparticles were transfected with the cells and tetra color One for the function of NADH as cell viability. b) Various types of T-MENDs rather than MENDs containing protaplex of positive (+ve) and negative (−ve) N/P ratios were approached for cell viability. Usually MENDs are well tolerated than a T-MEND of high lipid layers. Herein, various type of T-MENDs like R8 TMEND, R20/K5 (%) TMEND, R5/K20 (%) TMEND, R12.5/K12.5 (%) TMEND were transfected with the cells and cell viability as function of NADH function was evaluated.
Discussion

Previously we have mentioned that the DNA condensation/decondensation status with the respective cores are the crucial in transcription and here we have evaluated the condenser types of natural and synthetic origin (Akita et al., 2009). Polyrotaxane in rotaplex has the multi-valent interactions with DNA (Yui and Ooya, 2006) that caused the decondensation.

Figure 5. Comparative antigen presentation among the nanoparticles containing a rotaplex and a protaplex: a) Schematic diagram of an antigen presentation how it works. Nanoparticles, containing antigenic plasmid (SIINFEKL) epitope sequence were transfected in the cells and after endocytosis of the particles, the DNA particles appears in the nucleus and respective antigenic proteins containing SIINFEKL peptide would go to the surface for antigen presentation with T-Cell hybridoma. β-D-galactosidase and IL-2 were released by the T-Cell, which depends upon the intensity of interaction and activation of T-Cell. So when CPRG was incubated with the mixture, the enzyme released from the interaction will break CPRG and release Chlorophenol Red. And the system turns to brownish to red in color due to the presence of chlorophenol red, could be measured at 595 nm. b) An antigen presentation assay as per model were done transfecting the dendritic cells with the MENDs and T-MENDs, containing rotaplex and protaplex. Antigen presentation was measured as fold increase of absorbance measured at 595 nm under microplate reader, where JAWS-II/T-Cell was taken as one. Protaplex has got antigen presentation five times higher than that of a rotaplex mediated transfection.

Figure 6. Confocal microscopic study of fluorescently labeled rotaplex and protaplex loaded nanoparticles: Digital acquisition of QD- and rhodamine-derived signals. The emitted light derived from QD (ranging from 537 nm to 569 nm), and rhodamine (>580 nm) were corrected by META equipment, and then exhibited in green (G) and red (R) channels, respectively. a) A protaplex plasmid DNA fluorescently labeled with QD 545 and CX-Rhodamine, condensed with protamine. b) A rotaplex plasmid DNA fluorescently labeled with QD545 and rhodamine labeled PRX. Green indicates the clusters, where FRET was completely cancelled. Those indicated as red and yellow cluster where FRET was fully and partially occurred, respectively.
hampered in comparison to that of a protaplex, and made low transcription. This is why we found every case significantly low transgene expression (Fig. 2 and 3). We can consider the multivalent interactions with the binding sites of DNA, could be distinguished from non-specific interactions by their larger equilibrium binding constant (K), although mono-valent sugar-sugar and sugar-protein interactions could be comparatively quite smaller (Fig. 7).

Mamma et al. (Mammen et al., 1998a; Mammen et al., 1998b) and Yui and Ooya et al. (Mellman, 2013) showed that the free energy change in multivalent interactions is a function of total binding constant, \( K_N \), which is the ratio of \( K_a \) and \( K_d \), \( K_N = K_a / K_d \), \( K_a \) and \( K_d \) are the apparent binding rate constant and dissociation rate constant, respectively.

If we consider a hypothetical model of polyrotaxane, DNA, Protamine skeleton and their successive molecular image of interactions (Fig. 7), we will see that in multivalent interactions, hydrophobic and hydrophilic interactions among the polar groups and cyclic groups of cyclodextrin, where outer surface is hydrophilic and inner part is hydrophobic (due to having 18αCD), could be considered as a function of total accumulated apparent binding constant. The confocal image of highly condensed core in rotaplex, which is visualized as red due to QD-FRET, also showed a direct evidence of multivalent interactions (Fig. 6). This consequent value might be rather greater than those of its dissociation constant in reductive environment because of enormous interaction among the hydrophobic, ionic and other van der waal’s bonds. But if we see the DNA protamine complex it seems simply a hydrophilic interaction of ionic bonds rather than multiple interactions. And the hypothetical binding constant would be the smaller as per model (Fig. 7) than that of multivalent interactions, where apparent binding constant \( K_a \) is also the lesser than that of dissociation constant in reductive environment of the cell. However this model should be justified with more experiments and a few of our colleagues are now working with the complex.

From the confocal image in live cell of dendritic cell, many green signals with large area showed FRET cancellation, which means DNA are apart from the protamine, i.e. decondensed status already attained to be transcribed. And the consequent event reflected in high gene expression with lower toxicity.
When the GL3 plasmid was cloned to an antigenic plasmid containing SIINFEKL epitope and co-cultured with T-cell B32 hybridoma, packaged into the MEND and T-MEND system, antigen presentation showed rationally better transcription due to ease decondensation of the DNA-condenser complex of protaplex rather than that of a rotaplex (Fig. 5). Cell viability also suggested the ease interference with host DNA function in protaplex, where multi-valent interaction might cause host DNA malfunction, interfering with m-RNAs, essential for the cell viability. It supported the interaction of lipoplex and adenovirus in transgene expression. Previously Hama et al. (Nakamura et al., 2012) reported that lipoplex interfered mRNA, which made it inferior to that of adenovirus by many order of magnitudes in transgene expression.

Conclusion
Natural condenser of cationic amino acid enriched protein like protamine, which makes protaplex with DNA prefers better transgene expression, cell viability and respective antigen presentation in comparison to those of synthetic condenser like polyrotaxanes in rotaplex. Collectively, it could be commented that the interaction between a condenser and DNA is one of the potential factor, which affects transgene expression in immune cell like dendritic cell.

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Conflict of interest
The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Preparation of Fluorescently labeled protplex by a Condensation of double labeled plasmid DNA

Figure S2. a) Excitation wavelength of QD-545 at various Concentration, b) Before and after QD-labeled DNA condensation

Figure S3. Optimization of probe concentration for condensation due to QD-FRET: Pink colored curve showed the QD-FRET after condensation.