Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: The use of a nuclear transfer score

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Abstract **In the present study, the nuclear delivery of a green fluorescence protein (GFP)-encoding pDNA condensed by protamine was investigated in terms of trans-gene expression after cytoplasmic (*E*(cyt)) and nuclear (*E*(nuc)) microinjection. To compare the nuclear transfer process, a novel parameter; the nuclear transfer (NT) score was introduced. The *E*(cyt) value for protamine/pDNA particles increased in a charge ratio-dependent manner. The calculated NT score showed that this increase results from an enhancement in nuclear transfer efficiency, which was also quantitatively confirmed by a recently developed confocal image-assisted three-dimensionally integrated quantification (CIDIQ) method. Moreover, *E*(nuc) for protamine/pDNA particles was significantly higher than that for poly-L-lysine/pDNA particles, suggesting that pDNA, when condensed with protamine, is more accessible to intra-nuclear transcription. Collectively, protamine is an excellent DNA condenser, with bi-functional advantages: improvement in nuclear delivery and efficient intra-nuclear transcription.**
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1. Introduction

To develop a promising non-viral gene vector for clinical applications, an improvement in transfection efficiency is essential. Previous studies have clearly shown that various intracellular barriers, such as lysosomal degradation and nuclear membrane limit trans-gene expression. Lysosomal degradation can be avoided by utilizing various devices such as pH-sensitive fusogenic lipids, consisting of the dioleoyl phosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) [1–3], polycations that have proton sponge characteristics [4,5], and pH-sensitive membrane lytic peptides [6–9]. However, the nuclear membrane continues to be a barrier to transfection activity, especially in non-dividing cells. It has previously been reported that trans-gene expression is drastically enhanced in the M-phase when the nuclear membrane structure is diminished [10–13]. Therefore, an efficient system for the nuclear delivery of plasmid DNA (pDNA) is highly desired for the development of an artificial gene delivery system.

In an attempt to overcome the nuclear membrane barrier, pDNA is typically condensed with a cationic nuclear targeting signal peptide, such as cationic peptides modified with M9 derived from heterogeneous nuclear ribonucleoprotein-A1 [14], a TAT oligomer [15], a tetramer of the SV40 T-antigen-derived nuclear localization signal (NLS_{SV40}) [16], μ [17–19], and NLS_{SV40}-μ, a chimerical peptide of μ and NLS_{SV40} (Akita et al., submitted). In addition, protamine, a compound that has been approved by the US FDA is widely used as a DNA condenser ([20,21] and Moriguchi et al. submitted). As first proposed by Sorgi and collaborators [20], protamine has four possible NLS-like regions consisting of basic amino acid and proline or serine residues. The pre-condensation of pDNA with protamine actually enhanced the trans-gene expression mediated by cationic liposomes and/or polycations compared with poly-L-lysine (PLL), a commonly used pDNA condenser. However, based on these findings, it cannot be concluded that protamine is a useful device for the nuclear delivery of pDNA, since transfection activity is dependent on multiple intracellular events, including cellular uptake, endosomal escape, intracellular stability, nuclear transfer and intra-nuclear transcription. Cytoplasmic and nuclear microinjection proven to be a powerful technique for understanding the mechanism from the point of view of nuclear transfer and intra-nuclear transcription [22], since it enables the contribution of the efficiency of cellular uptake to be separated from and endosomal escape.

In the present study, we report on a quantitative evaluation of the nuclear transfer efficiency of protamine/pDNA particles in terms of the trans-gene expression of green fluorescence protein (GFP) after the cytoplasmic and nuclear microinjection of GFP-encoding pDNA, compared to that of PLL/pDNA particles, to investigate the potential of the protamine as a nuclear delivery device for pDNA.

2. Material and methods

2.1. General

HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). To prepare the reporter gene vector for the pDNA, an insert fragment encoding the EGFP was obtained by digesting *Eco*RI/*Not*I digestion of pEGFP-N1 (Clontech, Palo Alto, CA, USA) with *Eco*RI/*Not*I, followed by ligation to *Eco*RI/*Not*I digested site of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). pDNA samples were

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purified with a Qiagen (Valencia, CA) EndFree Mega kit. Protamine sulfate was obtained from Calbiochem (Ishikari, Japan) in purified form. Before use, protamine solution was filtered through the cellulose acetate filter (DISMIC-13cp: 0.2 μm pore size) obtained from Advantech (Chiba, Japan). PLL (MW = 27400) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tetramethylrhodamine-labeled dextran (RhoDex: MW: 70000) was purchased from Molecular probe (Eugene, OR, USA).

2.2. Preparation of the polycation/pDNA complex

For the condensation of pDNA, 20 μl of a pDNA solution (0.1 μg/μl in H_2O) was added dropwise to 100 μl of polycation solution under vortex for five times (totally 100 μl) at approximately 20-s intervals. The concentration of the protamine and PLL ($C_{polycation}$) at various charge ratios (+/−) was calculated using the following equation.

$$\text{Charge ratio} = \{C_{\text{polycation}} \times n_{\text{cation}}/\text{MW}_{\text{polycation}}\}/(C_{\text{DNA}}/\text{MW}_{\text{DNA}}),$$

where n_{cation} denotes the number of lysine and arginine residues in PLL and protamine, respectively. $MW_{polycation}$ and MW_{DNA} denote the molecular weight of the polycation (protamine: 4250, PLL: 27400) and one nucleotide (average: 308), respectively. C_{DNA} denotes the concentration of pDNA (0.1 μg/μl). As demonstrated previously, the polycation/pDNA complex was readily aggregated when condensed under a charge ratio of approximately 1.5 [23]. Therefore, charge ratio-dependent trans-gene expression was analyzed at the charge ratio above 2. The hydrodynamic diameter was measured by quasi-elastic light scattering by means of an electrophoresis light scattering spectrophotometer (ELS-8000, Otsuka electronics, Japan).

2.3. Microinjection study

The microinjection study was carried out, as described in the previous report [24] with minor modifications. Cells were seeded on the glass base dish 1 day (IWAKI, Osaka, Japan) before the microinjection. In this procedure, a semiautomatic injection system (Eppendolf transjector 5246, Hamburg, Germany) was attached to the Eppendolf micromanipulator 5171. Cytoplasmic and nuclear microinjections were performed under conditions of P_i = 50–70 hPa, P_c = 30 hPa and an injection time of 0.2 s. Just prior to injection, the pDNA, condensed with polycations, was diluted to 3.32 fmol/μl and 33.2 amol/μl with a 0.5% RhoDex/H_2O solution for cytoplasmic or nuclear microinjection, respectively. Under these conditions, 1000 copies and 10 copies of pDNA, respectively, were injected. At 24 h post-injection, GFP-expression was monitored by fluorescence microscopy, and the ratio of cells expressing GFP to RhoDex positive cells were calculated. In the nuclear microinjection, the rhodamine-positive cells were counted afterward, to avoid a situation in which the RhoDex diffuses into the cytosol during the 24 h incubation, which would lead to an underestimation of the number of nucleus-injected cells.

2.4. Quantitative evaluation of the nuclear delivery of protamine/pDNA particle by confocal images

For visualization of the pDNA after cytoplasmic injection, pDNA was labeled with rhodamine by the Label IT reagent (Panvera Corporation, Madison, WI, USA). At 1 h post-injection of the rhodamine-labeled pDNA condensed with protamine at charge ratios of 2 and 9, cells were incubated with 0.5 μM SYTO24 for 15 min to stain the nucleus. The cells were then washed three times with culture medium including 10% fetal calf serum. Fluorescence and bright field images were captured using a Zeiss Axiovert 200 inverted fluorescence microscope equipped with Achroplan 63×/0.95N objective (Carl Zeiss Co. Ltd.; Jena, Germany).

Quantification of the confocal images to evaluate the nuclear transfer efficiency was demonstrated by the recently developed confocal image-assisted three-dimensionally integrated quantification (CIDIQ) method [25]. 20 *Z*-series images were obtained from the bottom of the coverslip to the top of the cells and recorded by the Zeiss LSM510 on a PC. Each 8-bit TIFF image was transferred to Image-Pro Plus ver. 4.0 (Media Cybernetics Inc., Silver Spring, MD) to quantify the total brightness and pixel area of each r.o.i.. For the data analysis, the pixel areas of each cluster in the cytosol; s_i(cyt) and nucleus s_i(nuc) were separately summed in each XY plane, and are denoted as $S'_{Z=j}$(cyt) and $S'_{Z=j}$(nuc), respectively. The values of $S'_{Z=j}$(cyt) and $S'_{Z=j}$(nuc) in each X–Y plane were further summed through the all of *Z*-series of images, and are denoted as *S*(cyt) and *S*(nuc), respectively. These values represent the total amount of pDNA in the cytosol and the nucleus in an individual cell. The fractions of nuclear pDNA in the nucleus to the totally injected one; *F*(nuc), which represents the nuclear transfer efficiency were calculated using the equation below:

$$F(\text{nuc}) = \frac{S(\text{nuc})}{S(\text{cyt}) + S(\text{nuc})}.$$

3. Results

3.1. Physicochemical characterization of the polycation/pDNA particles

The particle sizes and *Z*-potentials of the pDNA/polycation particles condensed at charge ratios of 2 and 9 were characterized (Table 1). In the cases of both protamine and PLL, the particle sizes were approximately 65–90 nm at both charge ratios. The *Z*-potential of these particles increased depending on the charge ratios. In addition, the *Z*-potentials of the PLL/pDNA particles were significantly higher than for the protamine/pDNA particles.

3.2. Cytoplasmic and nuclear microinjections

We first evaluated the percent of GFP-positive cells obtained after cytoplasmic microinjection (*E*(cyt)). As shown in Fig. 1(a), the *E*(cyt) for protamine/pDNA particles at the high charge ratio of 9 (41.8% ± 4.9) was significantly higher than that at the low charge ratio of 2 (16.1% ± 3.8). Between the charge ratios of 2 and 9, *E*(cyt) was also increased in a charge ratio dependent manner (24.7% ± 4.5 and 38.8% ± 4.6 at a charge ratio of 4.5 and 6, respectively). At higher charge ratio of 12, *E*(cyt) was lower (33.5% ± 3.9) than that at a charge ratio of 9. In contrast, the *E*(cyt) values in PLL were not affected by the charge ratio (Fig. 1(a)). Therefore, the charge ratio-dependent increase in *E*(cyt) was specific for protamine/pDNA particles.

Since the maximum and minimum *E*(cyt) values occurred at charge ratios of 9 and 2, respectively, the percent of GFP-positive cells after nuclear microinjection (*E*(nuc)) were further compared at these charge ratios, in order to compare the intra-nuclear transcription efficiency. As shown in Fig. 1(b), the *E*(nuc) values for both protamine and PLL were not affected by the charge ratio. It is noteworthy that the *E*(nuc) values for the protamine/pDNA particles were higher than for PLL, suggesting that pDNA, when condensed with protamine, is more efficiently subject to the intra-nuclear transcription compared with that condensed with PLL.

Table 1
Physicochemical characteristics of polycation/pDNA particles

Sample name	Charge ratio	Size (nm)	Zeta potential (mV)
Protamine/pDNA	2.0	88.6 ± 9.0	8.9 ± 4.8
	9.0	76.3 ± 8.5	17.7 ± 4.0
Poly-L-lysine/pDNA	2.0	71.2 ± 3.5	27.6 ± 6.1
	9.0	65.7 ± 5.1	36.2 ± 9.3

Sizes and *Z*-potentials were measured an electrophoresis light scattering spectrophotometer. Data are represented as means ± S.D. of triplicate experiments.

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