Analysis of the Relationship between the Molecular Weight and Transfection Efficiency/Cytotoxicity of Poly-L-arginine on a Mammalian Cell Line

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The importance of the intracellular delivery of DNA and RNA has been highlighted for the therapeutic application as well as the fundamental research about the cell mechanism. After the introduction of the concept of gene therapy,[1] which involves the production of the therapeutic proteins by the delivery of DNA into cells and the discovery of siRNA,[2] which can control the translation of a specific protein, the development of the method for efficient and safe delivery of DNA and RNA has been one of the main objectives in the biological and medical science. Therefore, many chemists has been concentrated their efforts to synthesize the non-viral gene delivery carriers such as cationic lipids,[3] polymers,[4] and dendrimers[5] because they have various advantages over biological viral carriers such as easy large-scale production, low immune response, and high safety in the body.

Recently, the TAT sequence,[6] a nucleus localization signal (NLS) peptide sequence of the human immunodeficiency virus (HIV), has been applied to the improvement of efficiency of gene delivery carriers. With a help of the arginine-rich TAT sequence, various biopharmaceuticals including DNA could be delivered to cell nucleus, where the transcription of RNA from the DNA template occurs. Other similar arginine-rich peptide sequences were also reported to show significant nucleus localization of internal cargos in various non-viral gene carriers.[7] Moreover, the polymer or dendrimer modified with arginine[8] or guanidine[9] showed highly improved gene delivery efficiency.

Poly-L-arginine (PArg) (Figure 1A) was also applied as the gene delivery carriers based on the reports above.[10] However, although the characteristics of its lysine derivative, poly-L-lysine (PLL) (Figure 1A), was examined profoundly in terms of the molecular weight, transfection efficiency, and cytotoxicity,[11] the comprehensive research about the relationship between the molecular weight of PArg and its biological activity has not been performed yet. Here, we wish to show the advantages of PArg over PLL through the examination of the physicochemical characteristics and the biological activity.

PArg polymers with three different molecular weights were purchased from Sigma (St. Louis, MO). Although the molecular weights are informed from the distributor, the accuracy is not adequate for the systematic research. Therefore, we re-confirmed the molecular weights by multi angle laser light scattering (MALLS) method. As shown in Table 1, the M_w (weight-averaged molecular weight) values of 10 kDa, 41 kDa, and 83 kDa were measured by MALLS, of which accuracy and precision are quite different from the values of the distributor. The molecular weights of PArg polymers are between 10 kDa and 100 kDa, the range of the molecular weights of most of polymers for gene delivery. The PD (M_w/M_n) of the 10 kDa PArg was 1.838, which showed moderately narrow distribution, and the PDs of 41 kDa and 83 kDa PArgs were 1.140 and 1.042, respectively, which showed narrow distribution.

The transfection efficiencies on human embryonic kidney (HEK) 293 cells of PArg polymers were compared according to the variation of their molecular weights by using luciferase plsamid. The high-molecular-weight PArg polymers (41 kDa and 83 kDa) showed about 100 times higher transfection efficiency than the low-molecular-weight PArg (10 kDa) (Figure 1B). The transfection efficiency was dependent upon

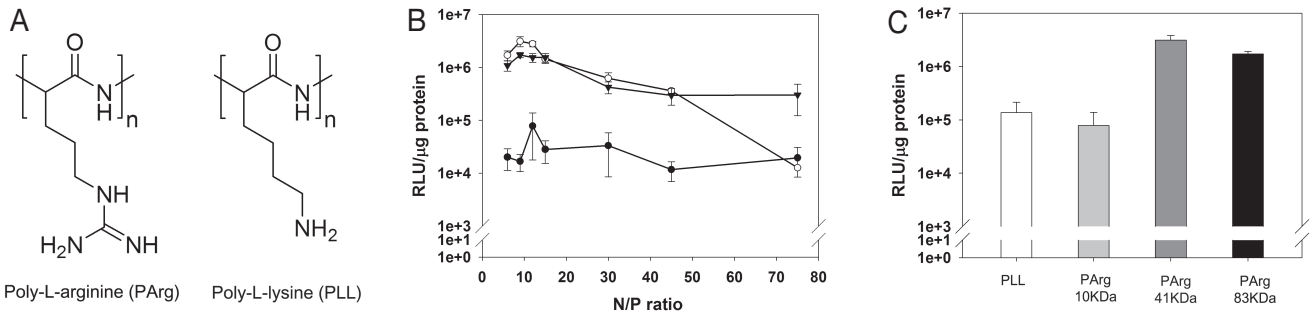

Figure 1. (A) The structures of PArg and PLL. (B) N/P ratio-transfection efficiency relationship of PArg 10 kDa (●), 41 kDa (○), and 83 kDa (▾). (C) The comparison of the transfection efficiency between PLL and PArg polymers at their optimal ratios. Each error bar represents standard deviation (± S. D.).

Table 1. The molecular weight measurement of PArg and PLL

	M_w [a]	M_w [b]	M_n [b]	PD (M_w/M_n) [b]	dn/dc (mL/g)
PArg 10kDa	5,000-15,000	10,360	5,639	1.838	0.1968
PArg 41kDa	15,000-70,000	41,340	36,280	1.140	0.1422
PArg 83kDa	> 70,000	82,840	79,520	1.042	0.1499
PLL	30,000-70,000	46,760	24,530	1.906	0.1769

[a]referenced from the producer catalogues. [b]measured by MALLS.

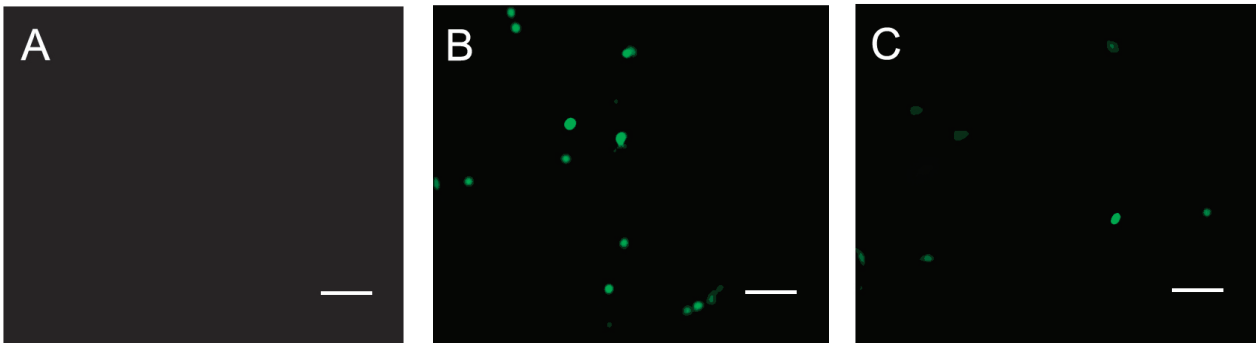

Figure 2. Fluorescence microscopic images of HEK 293 cells transfected with the polyplex between pEGFP and (A) PArg 10 kDa, (B) 41 kDa, and (C) 83 kDa. Each bar represents 100 μm.

the guanidine (N) to phosphate (P) ratios. All three PArg polymers showed their maximum transfection efficiency at the N/P ratio of around 10. The transfection efficiency of PArg was also compared with that of PLL (Figure 1C). Because it was reported that the transfection efficiency of the high-molecular-weight PLL (over 20 kDa) showed significantly higher transfection efficiency than that of the low-molecular-weight PLL,[11] we chose the 47 kDa PLL with a similar molecular weight with 41 kDa PArg, as a control. At their optimal N/P ratios, the PArg (41 kDa) showed over 10 times higher transfection efficiency than PLL.

The difference of the transfection efficiency depending upon the molecular weight can be also confirmed using the plasmid coding enhanced green fluorescence protein (EGFP) gene. The 293 celles transfected with PArg 10 kDa showed almost no green fluorescence (Figure 2A), whereas, the cells transfected with PArg 41 kDa showed bright green fluorescence (Figure 2B). The intensity of the green fluorescence in the cells transfected with Arg 83 kDa was slightly weaker (Figure 2C).

Next, we have compared the stability of the polymer-DNA complex (polyplex) of PArg and PLL. The polyplexes at the N/P ratio of 9, the optimal ratio for the transfection, were treated with various amounts of heparin, an anionic macromolecule to dissociate DNA from polyplexes by competing with DNA through electrostatic interaction.[12] The stability of the polyplex against the addition of heparin was analyzed by agarose gel retardation assay (Figure 3). The PLL polyplex released DNA at the 1.0 mg/mL of heparin, whereas, the PArg polyplex show almost no release up to 1.5 mg/mL of heparin. The higher basicity of the guanidine (pKa = 12.5) in PArg over the amine (pKa = 10.7) in PLL and the additional hydrogen bonds between the phosphates in DNA and guanidines in PArg are probably the main reasons for the enhanced stability of PArg polyplex. Although the main reason of the higher transfection efficiency of PArg over PLL was the plasma membrane penetrating activity and nucleus localization activity of PArg,[13] the higher stability of the PArg polyplex against anionic molecules in the extracellular matrix could also support the higher efficiency.

Finally, the cytotoxicities of PArg polymers were compared according to the variation of molecular weight (Figure 4). Similarly to other cationic polymers for the gene delivery, the higher-molecular-weight PArg showed higher cytotoxicity. PArg 10 kDa showed almost no cytotoxicity even at 100 μg/mL. However, the IC_{50} value of PArg 41 kDa is around 100 μg/mL, and that of PArg 83 kDa is around 70 μg/mL. Considering that the IC_{50} value of PLL was reported as below 20 μg/mL,[11] the PArg can be promising as a nontoxic and efficient gene delivery carrier. Selection of the optimal molecular weight among PArg polymers for both high transfection efficiency and low cytotoxicity should be essential for the successful gene delivery.

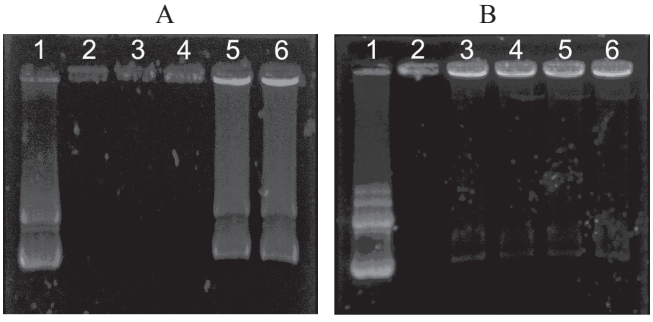

Figure 3. The stability of the PLL (A) and PArg (B) polyplexes against the addition of heparin. The lane 1, 2, 3, 4, 5, and 6 represent DNA only, polyplex only, polyplex added by 0.3 mg/mL heparin, polyplex added by 0.7 mg/mL heparin, polyplex added by 1.0 mg/mL heparin, and polyplex added by 1.5 mg/mL heparin, respectively.

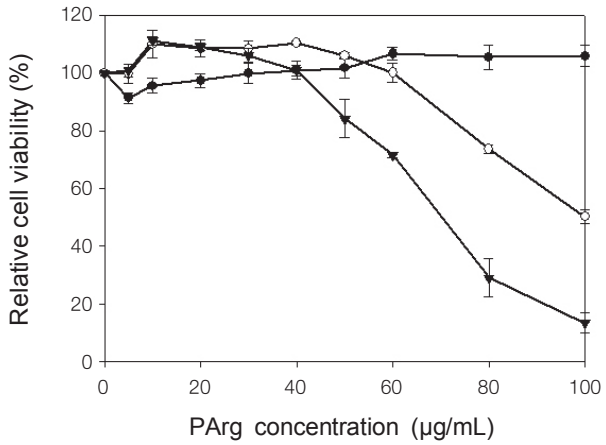

Figure 4. The cytotoxicity on 293 cells of PArg 10 kDa (●), 41 kDa (○), and 83 kDa (▼). Each error bar represents standard deviation (± S. D.).

In summary, we examined the transfection efficiency and cytotoxicity of PArg polymers and confirmed the relationship between the molecular weight and the potential for the DNA delivery. PArg has also been applied for the specific repression of the protein production by siRNA delivery.[14] By controlling and selection of the optimal molecular weight for biological use as reported here, PArg would have great potential as a biocompatible and functional polymers for DNA and siRNA delivery in the medical field as well as the biological field.

Experimental Section

Molecular weight measurement of PArg by MALLS. Each molecular weight was determined by MALLS in combination with size-exclusion chromatography (SEC).[15] The SEC system included a P680 HPLC pump from Dionex Corporation (USA). Polymer samples were detected by a three-angle laser-light-scattering detector (miniDAWN Tristar, 30 mW GaAs laser, 690 nm, K5 cell) and an interferometric refractometer (Optilab DSP, P10 cell) from Wyatt Technologies (USA). A 1% formic acid aqueous solution was used as an eluent after filtration through a 22-nm filter and degassing. The d*n*/d*c* value for each polymer was also measured by the same interferometric refractometer.

Transfection assay in the HEK 293 cell line. The HEK 293 cells were seeded at a density of 3×10^4 cells/well in 24-well plates with 600 μL of Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science, USA) containing 10% Fatal Bovine Serum (FBS) (GIBCO, USA) and grown to 70-80% confluence for one day. The cells in each well was treated with polyplex solution containing 2 μg of pCN-Luci plasmid DNA at various N/P ratios for 4 h at 37 °C.[16] Following 4 h-treatment of polyplexes, the medium was replaced by 600 μL of fresh medium containing 10% FBS. After an additional incubation for 2 days, the growth medium was removed. The cells were rinsed with 240 μL of phosphate buffered saline (PBS) and lysed for 30 min at room temperature by using 120 μL of reporter lysis buffer (Promega, USA). The luciferase activity was measured using a LB 9507 luminometer (Berthold, Germany), and the protein content was measured by using a Micro BCA assay reagent kit (Pierce, Rockford, IL). The same transfection method was used for the pEGFP-C2 DNA delivery. The fluorescent images were observed and recorded with Axiovert 200M Carl Zeiss microscope.

Polyplex stability assay. The polyplex solutions (10 mM Hepes, 1 mM NaCl, pH 7.4) containing 1 μg DNA were prepared at an N/P ratio of 9. After 30 min incubation at room temperature for the polyplex formation, various amounts of heparin solution was added to the polyplex solutions. After an additional 30 min-incubation, the samples were electrophoresed on a 0.7% (w/v) agarose gel and stained with an ethidium bromide solution (0.5 μg/mL). The location of the DNA was analyzed on a UV illuminator.

Cytotoxicity assay. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cytotoxicity. HEK 293 cells were seeded in 96-well tissue culture plates at a density of 8×10^3 cells/well in 90 μL DMEM medium containing 10% FBS. The cells achieving 70-80% confluence after 24 h were exposed to 10 μL of various concentrations of the polymer solutions for 4 h. 26 μL of the solution of MTT (Sigma, USA) (2 mg/mL in PBS) was added to each well. After an additional 2 h-incubation at 37 °C, the media was removed, and the resulting formazan was dissolved with 150 μL of dimethylsulfoxide (DMSO). The absorbance was measured at 570 nm by using a microplate reader (Molecular Devices Company, USA). The relative cell viability was calculated as a percent absorbance to untreated control cells.

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