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Potential of D-Octaarginine-Linked Polymers as an in Vitro Transfection Tool for Biomolecules

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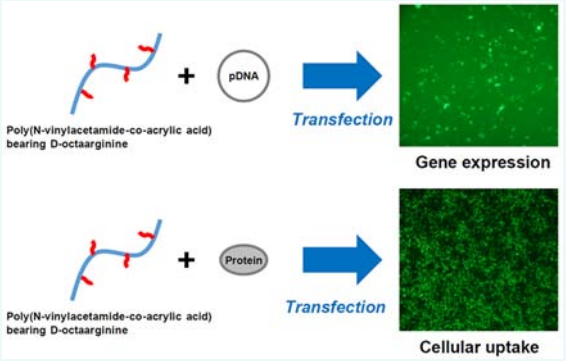
ABSTRACT: We have been investigating the potential use of cell-penetrating peptide-linked polymers as a novel penetration enhancer. Since previous in vivo studies demonstrated that poly(*N*-vinylacetamide-*co*-acrylic acid) bearing D-octaarginine, a typical cell-penetrating peptide, enhanced membrane permeation of biomolecules, its potential as an in vitro transfection tool was evaluated in this study. A plasmid DNA encoding green fluorescent protein (pGFP-C1), *β*-galactosidase, and bovine serum albumin (BSA) were used as model biomolecules. Anionic pGFP-C1 interacted electrostatically with cationic D-octaarginine-linked polymers. When the ratio of mass concentration of polymers to that of pGFP-C1 reached 2.5, complexes whose size and zeta potential were approximately 200 nm and 15 mV, respectively, were obtained. GFP expression was observed in cells incubated with complexes prepared under conditions in which the polymer/pDNA concentration ratio exceeded 2.5. The expression level elevated with an increase in the concentration ratio, but physicochemical properties of the complexes remained unchanged. Results suggested that free polymers contributed to pGFP-C1 internalization. Another cell study demonstrated that *β*-galactosidase premixed with polymers was taken up into cells in its active tetrameric form. Similar electrostatic interaction-driven complex formation was observed for BSA charged negatively in neutral solution. However, it appeared that the internalization processes of BSA differed from those of pGFP-C1. A mass concentration-dependent increase in internalized BSA was observed, irrespective of the polymer/protein concentration ratio. Due to frail interactions, polymers that were released from the complexes and subsequently immobilized on cell membranes might also contribute to membrane permeation of BSA.



INTRODUCTION

The development of biomolecules such as proteins and oligonucleotides is globally competitive. Such biomolecules possess highly specific functions to target molecules. Their performance is mainly observed in the cytoplasm and nucleus of cells in which target molecules are present, while there are biomolecules that recognize their target molecules on cell membranes such as antibodies.[1−4] Biomolecules are promising as a new class of active pharmaceutical ingredients, but there are issues to be solved for their universal use in clinics. One such issue is the low membrane permeability of biomolecules, that is attributed to their large molecular weights and/or hydrophilicity. Technologies that enhance their permeability through cell membranes and deliver biomolecules to target molecules inside cells are strongly desired.[5−7]

Cell-penetrating peptides rich in basic amino acids such as arginine have recently emerged as a powerful tool to overcome the challenge of penetration enhancement of biomolecules, which are not very membrane permeable or completely membrane impermeable.[8−11] The most typical cell-penetrating peptides are the human immunodeficiency virus (HIV)-1 Tat protein (48−60), penetratin, and oligoarginines. These cationic oligopeptides are effectively taken up into cells through their high affinity to cell membranes.[12−14] A mechanism on the cellular uptake remains controversial; however, macropinocytosis through reorganization of filamentous actin-binding proteins has mainly been discussed.[14−16] Guanidine moieties in arginine, which compose part of the cell-penetrating peptides, are essential for efficient internalization of the peptides. Macropinocytosis is initiated through guanidine moiety-induced electrostatic interactions between the peptides and membrane-associated proteoglycans such as heparan sulfate.[15,17]

A couple of strategies have mainly been investigated with the aim of improving penetration abilities of insufficiently membrane permeable or membrane-impermeable biomolecules: conjugation of biomolecules with cell-penetrating peptides and surface modification of biomolecule-incorporated

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vehicles with the peptides.[18−21] In both strategies, cell-penetrating peptides are taken up into cells along with the peptide-bound biomolecules or vehicles. Alternatively, we designed cell-penetrating peptide-linked polymers with a unique strategy in which penetration enhancement of biomolecules is not always concomitant with cellular uptake of the peptides bound to polymers.[22−25] We hypothesized that recognition of peptidyl branches in the polymer backbone triggers macropinocytosis on the membrane surface of cells exposed to a mixture of biomolecules and cell-penetrating peptide-linked polymers. Since macropinocytosis proceeds extensively in the multiple peptide access points of the single-stranded polymers, cells fail in uptake of peptides anchored chemically to the polymeric platform. Instead, a series of macropinocytotic processes results in the incidental uptake of biomolecules located in the periphery of the cell membrane-recognized peptidyl branches.

Our hypothesis has been validated in numerous in vivo studies using poly(*N*-vinylacetamide-*co*-acrylic acid) (PNVA-*co*-AA) bearing oligoarginines. The hypoglycemic effects of antidiabetic peptide drugs such as insulin and exendin-4 were significantly enhanced when nasally coadministered with PNVA-*co*-AA bearing D-octaarginine (Figure 1), which is 8

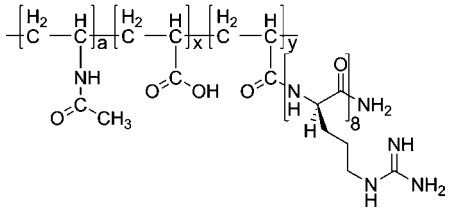

Figure 1. Chemical structure of PNVA-*co*-AA bearing D-octaarginine.

arginine residues with D-configuration, in mice. On the other hand, the effect disappeared when D-octaarginine-linked PNVA-*co*-AA was substituted with either D-octaarginine alone or a mixture of unlinked D-octaarginine and PNVA-*co*-AA. Results demonstrated that only D-octaarginine anchored to the polymeric platform enhanced membrane permeation of the peptide drugs.[22] Other mice experiments identified D-octaarginine-linked PNVA-*co*-AA as a potential candidate of antigen carriers for mucosal vaccine delivery, which induces humoral immunity on the mucosal surface and in systemic circulation when nasally coadministered with protein-based antigens such as influenza virus vaccines.[24,25]

We have valuable evidence for the potential use of oligoarginine-linked polymers as an in vivo delivery tool that enhances membrane permeation of biomolecules including vaccine antigens. However, there is limited data on the feasibility of improving the in vitro permeability of insufficiently membrane permeable or membrane-impermeable molecules by oligoarginine-linked polymers. Only atenolol and 5(6)-carboxyfluorescein, which are chemical substances with a low molecular weight, and fluorescein isothiocyanate (FITC)-conjugated dextran with a molecular weight of 4 kDa, which is a derivative of polysaccharides, have been used as model compounds to study the mechanism on enhancement of membrane permeation.[23] In the present study, we evaluated the potential use of D-octaarginine-linked PNVA-*co*-AA as an in vitro transfection tool of biomolecules with larger molecular weights using plasmid DNA (pDNA) and proteins. Through the study, data were expressed as a function of the ratio of mass concentration (μg/mL) of the polymer to that of the biomolecule.

RESULTS

Interactions between Biomolecules and D-Octaarginine-Linked PNVA-*co*-AA. *Plasmid DNA.* pGFP-C1, which encodes green fluorescent protein (GFP), was used as a model pDNA. The interactions between pGFP-C1 and D-octaarginine-linked PNVA-*co*-AA were first evaluated by agarose gel electrophoresis. As shown in Figure 2A, bands of the pDNA

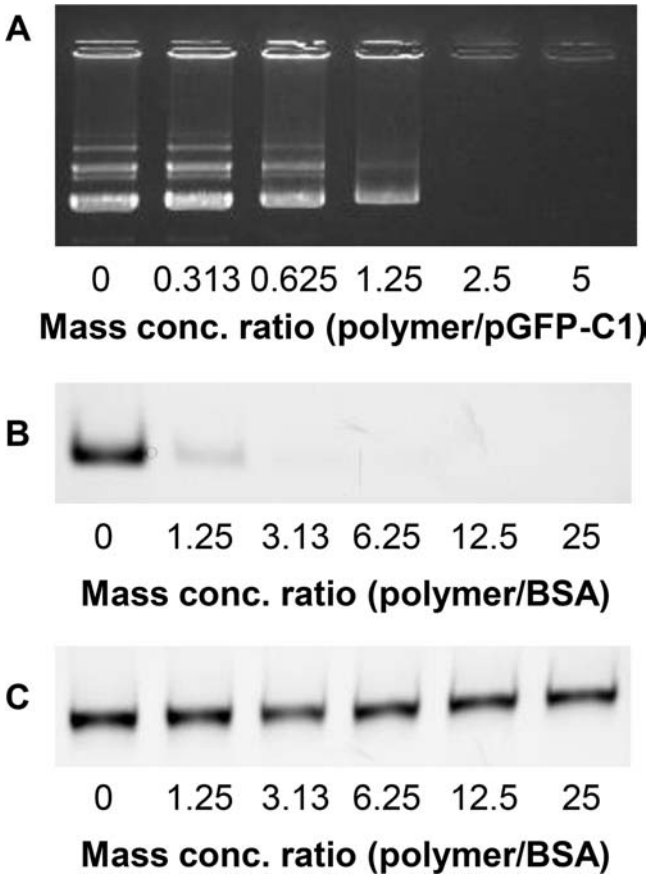

Figure 2. Gel electrophoresis of biomolecules mixed with or without D-octaarginine-linked PNVA-*co*-AA. (A) Agarose gel electrophoresis with ethidium bromide exclusion assay was performed for a mixture of pGFP-C1 and the polymer. Before loading onto the gel, the mixture was left for 15 min at room temperature. Electrophoresis of pGFP-C1 (1 μg) was carried out at 100 V for 15 min in Tris-acetate-EDTA buffer. (B) Native PAGE electrophoresis was performed for a mixture of FITC-BSA and the polymer which was prepared in a manner similar to that described above. Electrophoresis of FITC-BSA (0.05 μg) was carried out at 200 V for 60 min in Tris-borate-EDTA buffer. (C) SDS-PAGE was performed for a mixture of FITC-BSA and the polymer in the same manner described above.

were clearly detected under polymer-free conditions. Bands with reduced intensity were observed for the pDNA samples mixed with a small amount of polymers. Bands disappeared when the mass concentration ratio of the polymer to the pDNA was set to 2.5. No band was observed through further elevation of the concentration ratio.

Dynamic light scattering spectrophotometry revealed that particulate formation resulted from the interactions of pGFP-C1 with D-octaarginine-linked PNVA-*co*-AA (Figure 3A).

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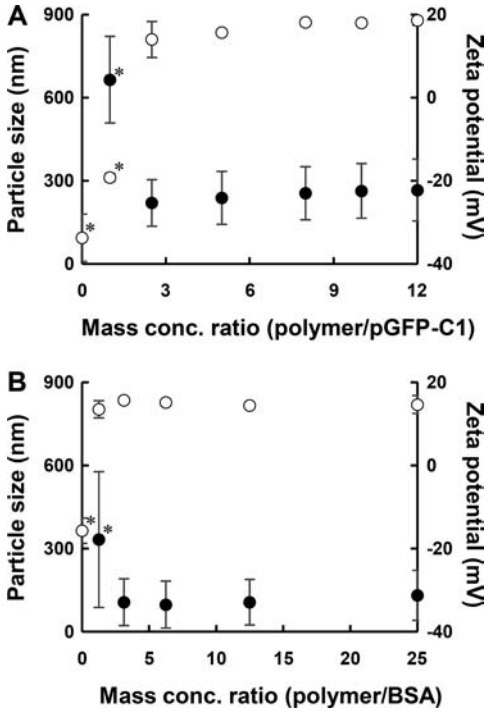

Figure 3. Size (●) and zeta potential (○) of particles formed through interactions between biomolecules (A: pGFP-C1, B: BSA) and D-octaarginine-linked PNVA-*co*-AA in PBS at 25 °C. Both physicochemical properties were measured by dynamic light scattering spectrophotometry. Concentrations of pGFP-C1 and BSA were constantly 10 μg/mL and 20 μg/mL, respectively. Measurements were made on mixtures ranging in the polymer concentration from 0 to 120 μg/mL for pGFP-C1 and 0 to 500 μg/mL for BSA. Before measurements, the mixture was left for 15 min at room temperature. Each value represents mean ± SD (particle size: 70 times measurement, zeta potential: 3 experiments). * indicates statistically significant difference ($p < 0.05$) from either the polymer/pGFP-C1 ratio of 2.5 (A) or the polymer/BSA ratio of 3.13 (B).

Particles with an average size of approximately 650 nm were observed when the pDNA was mixed with an equal amount of polymer. They were negatively charged, but the absolute value was less than that of intact pGFP-C1. When the mass concentration ratio of the polymer to the pDNA was set to 2.5, the particle size was reduced to approximately 200 nm and the zeta potential elevated to approximately 15 mV on an average. Neither of physicochemical properties changed significantly through a further increase of the concentration ratio.

Protein. Bovine serum albumin (BSA), with or without a FITC probe, was used as a model protein. Native polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed to evaluate the interactions between FITC-BSA and D-octaarginine-linked PNVA-*co*-AA (Figure 2B,C). A single fluorescent band of FITC-BSA was detected on a native polyacrylamide gel under polymer-free conditions. The band intensity was reduced when FITC-BSA was mixed with the polymer at a mass concentration ratio of 1.25 (polymer/FITC-BSA). The fluorescent band disappeared when the concentration ratio was set to 3.13 or above. On the other hand, the FITC-BSA band was stably expressed on a polyacrylamide gel containing SDS, regardless of the change in polymer concentration.

As shown in Figure 3B, similar particulate formation was observed for mixtures of FITC-free BSA and D-octaarginine-linked PNVA-*co*-AA. The average size of particles was constantly ca. 100 nm when the mass concentration ratio of the polymer to BSA was set to 3.13 or above, at which the FITC-BSA band was not detected on the native polyacrylamide gel. Constant positive charges of particles were observed in polymer-containing PBS, although BSA was negatively charged under polymer-free conditions.

Cellular Uptake of Biomolecules Mixed with D-Octaarginine-Linked PNVA-*co*-AA. *Plasmid DNA.* Cellular uptake of pGFP-C1 was first evaluated by fluorescence microphotography. HeLa cells, which is a human uterocervical carcinoma cell line, were used. As shown in Figure 4A, clear green fluorescence was observed when cells were incubated with a mixture of pGFP-C1 and D-octaarginine-linked PNVA-*co*-AA whose mass concentration ratio (polymer/pGFP-C1) was 5. On the other hand, no green fluorescence was detected

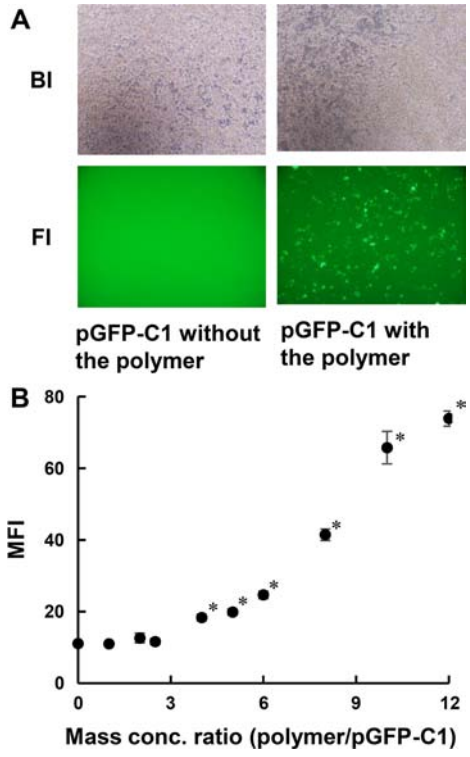

Figure 4. GFP expression in HeLa cells incubated with pGFP-C1. (A) Fluorescence images (FI) and bright field images (BI) of HeLa cells (magnification: ×100; excitation: 470−495 nm; emission: 510−550 nm; exposure for the FI: 1/5 of a second; exposure for the BI: 1/300 of a second). The BI was obtained at the same site as the FI. The pDNA (5 μg/mL) was mixed with or without D-octaarginine-linked PNVA-*co*-AA (0 or 25 μg/mL) and the mixture was then left for 15 min at room temperature. Four-hour incubation of HeLa cells with the mixture was followed by 68-h incubation in the polymer/pDNA-free culture medium. Temperature was set to 37 °C. (B) Mean fluorescence intensity (MFI) of HeLa cells measured by flow cytometry. HeLa cells were incubated with pGFP-C1 by means of the same procedure as described above (pDNA concentration: 2 μg/mL, polymer concentration: 0−24 μg/mL). Excitation and emission wavelengths were set to 488 and 525 nm, respectively. Each value represents mean ± SD of 3 experiments. * denotes statistically significant difference ($p < 0.05$) from polymer-free conditions.

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in cells incubated with the pDNA under polymer-free conditions.

Flow cytometry was next performed to evaluate the cellular uptake quantitatively (Figure 4B). The mean fluorescence intensity (MFI) of HeLa cells was approximately 10 when they were incubated with pGFP-C1 alone. This value was similar to that observed in the pDNA-free medium (10.3 ± 0.4). This low MFI remained unchanged when a small amount of D-octaarginine-linked PNVA-*co*-AA was dissolved in the medium used for the initial 4 h incubation. When the concentration ratio of the polymer to the pDNA exceeded 2.5, a ratio-dependent increase in the MFI was observed.

Protein. Fluorescence microphotography was also performed to evaluate cellular uptake of FITC-BSA (Figure 5A). FITC-BSA-derived green fluorescence was observed in HeLa cells incubated with a mixture of FTC-BSA and D-octaarginine-linked PNVA-*co*-AA at 37 °C, while removal of the polymer resulted in disappearance of the fluorescence. No fluorescence was observed in cells incubated with FITC-BSA at 4 °C even when the polymer was present in the medium, indicating that the fluorescent protein was taken up into cells via endocytic pathways including macropinocytosis. As shown in Figure 5B, this observation was supported quantitatively by the MFI of the respective cells measured using a fluorescence-activated cell sorter (the MFI of HeLa cells incubated with a mixture of FITC-BSA and the polymer at 4 °C was statistically larger than that obtained under the corresponding polymer-free conditions, but the statistical significance would not make any sense because the difference was extremely small when compared with that observed at 37 °C).

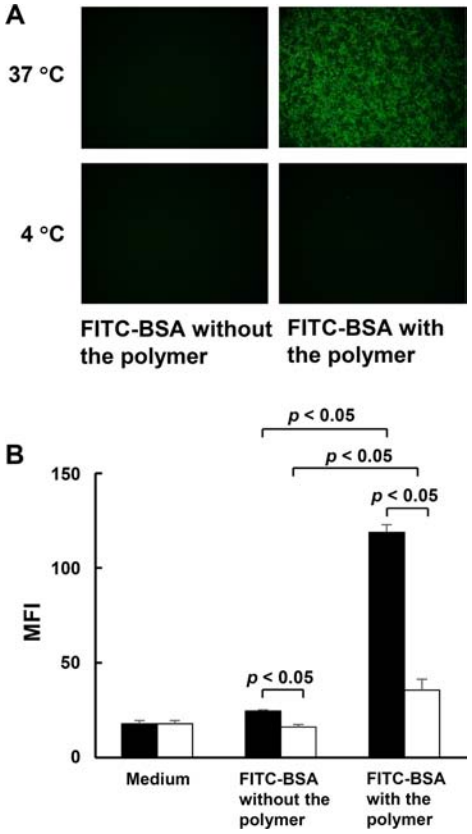

Figure 5. Fluorescence images (A) (magnification: ×100; excitation: 470−495 nm; emission: 510−550 nm; exposure: 1/20 of a second) and the flow cytometry-estimated MFI (B) (excitation: 488 nm; emission: 530 nm) of HeLa cells incubated with FITC-BSA (5 μg/mL) which was mixed with or without D-octaarginine-linked PNVA-*co*-AA (0 or 12.5 μg/mL) at either 37 or 4 °C. Before incubation, the mixture was left for 15 min at room temperature. Black and white bars in (B) correspond to the MFI of cells incubated at 37 and 4 °C, respectively. Each value represents mean ± SD of 3 experiments.

The MFI measurement was subsequently performed for HeLa and Chinese Hamster Ovary (CHO) cells under various mass concentrations of D-octaarginine-linked PNVA-*co*-AA and FITC-BSA. As shown in Figure 6A, the MFI of HeLa cells was approximately 10 when they were incubated with FITC-BSA alone. This value was similar to that observed in the protein-free medium (11.3 ± 1.9), indicating that there was insignificant nonspecific interaction between FITC-BSA and HeLa cells. The MFI of HeLa cells incubated with a mixture of FITC-BSA and D-octaarginine-linked PNVA-*co*-AA was constantly higher than that obtained under polymer-free conditions. The MFI increased in a polymer concentration-dependent manner under constant FITC-BSA concentration; however, the MFI increase was suppressed with the highest concentration of polymers. The MFI measurement was next made under constant polymer concentration with different FITC-BSA concentrations (Figure 6B). The MFI increased with an elevation of FITC-BSA concentration which resulted in

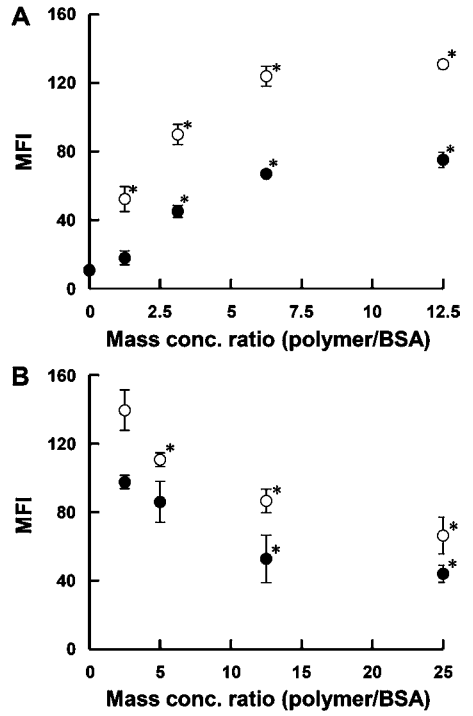

Figure 6. Cellular uptake of FITC-BSA mixed with or without D-octaarginine-linked PNVA-*co*-AA. (A) Flow cytometry-estimated MFI of HeLa (●) and CHO (○) cells at 1 h after incubation under constant concentration of FITC-BSA (1 μg/mL) with different concentrations of the polymer (0−12.5 μg/mL). (B) The MFI of each cell at 1 h after incubation under constant concentration of the polymer (12.5 μg/mL) with different concentrations of FITC-BSA (0.5−5 μg/mL). Before 1 h incubation in both experiments, the mixture was left for 15 min at room temperature. Temperature was set to 37 °C. Excitation and emission wavelengths were set to 488 and 530 nm, respectively. Each value represents mean ± SD of 3 experiments. * indicates statistically significant difference ($p < 0.05$) from either polymer-free conditions (A) or polymer/BSA ratio of 2.5 (B).

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a reduction of the concentration ratio (polymer/protein). When HeLa cells were substituted with CHO cells, a similar trend was observed (the MFI of CHO cells incubated under polymer/FITC-BSA-free conditions: 10.3 ± 1.2); however, FITC-BSA uptake occurred at a higher level in CHO cells than HeLa cells.

D-Octaarginine-linked PNVA-*co*-AA-induced cellular uptake of β-galactosidase, which is often used as a reporter protein, was finally evaluated through assay of its enzymatic activities in cell lysates. As shown in Figure 7, the mean absorbance observed

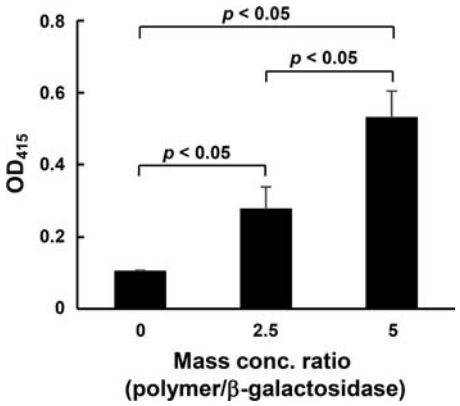

Figure 7. Cellular uptake of β-galactosidase mixed with or without D-octaarginine-linked PNVA-*co*-AA. The enzyme (5 μg/mL) was mixed with or without D-octaarginine-linked PNVA-*co*-AA (0–25 μg/mL) and the mixture was then left for 15 min at room temperature. HeLa cells were incubated with the mixture for 1 h at 37 °C. After cells were lysed, the absorbance of the supernatant obtained through centrifugation of the lysed solution was measured at 415 nm to estimate enzymatic activities of β-galactosidase taken up into cells. Each value represents mean ± SD of 3 experiments.

under polymer-free conditions was approximately 0.1. This control value significantly increased when HeLa cells were incubated with a mixture of β-galactosidase and the polymers whose mass concentration ratio (polymer/protein) was 2.5. The absorbance, from which the control value was subtracted, doubled through a 2-fold elevation of the concentration ratio.

DISCUSSION

Many studies on in vitro transfection have focused on nucleic acids such as genes, pDNA, and small interfering RNA. Since nucleic acids possess phosphoric acid esters, they are negatively charged without exception. Many cationic compounds that interact electrostatically with anionic nucleic acids have been investigated, and some of them, e.g., cationic lipids such as lipofectamine, are commercially available as in vitro transfection reagents. Complexes with a net positive charge are formed through electrostatic interactions. This phenomenon is often associated with particulate formation. In the conventional technique, electrostatic interaction-based formation is a prerequisite for cellular uptake of insufficiently membrane permeable or membrane-impermeable biomolecules, and internalization of the resulting complexes is accompanied by that of delivery tools which are counterparts of biomolecules.[26,27]

The most important finding in the present study is that membrane-impermeable pDNA and proteins were successfully taken up into cells in their active forms when incubated with D-octaarginine-linked PNVA-*co*-AA. The observed GFP expression demonstrated that pGFP-C1, whose molecular weight is 2912 kDa (4728 bp), was internalized into cells during the initial 4 h incubation with polymers and that GFP was expressed in the cytoplasm through transcription and translation of the internalized pDNA during the subsequent 68 h incubation without polymers (Figure 4). It is generally accepted that pDNA must overcome numerous intracellular barriers for successful transgene expression. Cellular uptake is one of such barriers, and escape of pDNA from endosomes and/or its nuclear transport should be considered subsequent barriers for internalized pDNA. We currently do not have any evidence that supports how pGFP-C1 is translocated to the cell nucleus. Oligoarginine-linked polymers were designed as a tool that would enhance the permeability of bioactive molecules through cell membranes; however, they might also participate in intracellular events of biomolecules. The molecular weight of β-galactosidase is 116 kDa and its tetrameric structure is essential for expression of enzymatic activities.[28] Figure 7 demonstrated that tetramers of β-galactosidase with a molecular weight of 464 kDa were taken up into cells through incubation with D-octaarginine-linked PNVA-*co*-AA. Not only did our polymers enhance in vitro membrane permeation of chemical substances with low molecular weights,[23] but they also delivered membrane-impermeable biomolecules with large molecular weights into cells.

We previously evaluated the in vitro performance of D-octaarginine-linked PNVA-*co*-AA physically mixed with insufficiently membrane permeable or membrane-impermeable molecules.[23] Anionic 5(6)-carboxyfluorescein (CF) was taken up into Caco-2 cells, which is a human colorectal adenocarcinoma cell line, through incubation with polymers. Polymer-induced CF uptake remained constantly high for the duration of a 120 min experiment, even though Zaro and Shen reported that cellular uptake of intact oligoarginines terminated within 15 min.[29] The CF uptake was significantly suppressed by an inhibitor of macropinocytosis. Confocal laser scanning microscopy revealed that the outline of Caco-2 cells was stained with red fluorescence after incubation with D-octaarginine-linked PNVA-*co*-AA bearing rhodamine red. Results indicated that D-octaarginine-linked polymers remained on cell membranes while CF was continuously internalized into cells mainly via macropinocytosis repeated for the individual peptidyl branches in the polymer backbone, as originally hypothesized.[22] Internalization of cationic atenolol and nonionic FITC-dextran were also observed in Caco-2 cells incubated with D-octaarginine-linked polymers. The charge-independent cellular uptake indicated that electrostatic interactions between oligoarginine-linked polymers and bioactive molecules were not always required for our technology.

Electrophoretic analysis revealed that pGFP-C1 interacted with D-octaarginine-linked PNVA-*co*-AA (Figure 2A). No bands of the pDNA were detected when the mass concentration ratio (polymer/pDNA) was 2.5 or above. As shown in Figure 3A, the size and zeta potential of the interaction-based particles were stable when the concentration ratio was set to the values mentioned above. It was likely that anionic pGFP-C1 became saturated with cationic polymers at a mass ratio of 2.5 (its N/P ratio was calculated to be approximately 3 on the basis of molecular weights of D-octaarginine-linked polymers and the linkage level of the oligopeptides in the polymer backbone) and that free polymers were present in solution where the ratio was more than 2.5. Similar phenomena were observed for BSA (Figures 2B and 3B). Native-PAGE indicated that the border of

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the mass concentration ratio (polymer/protein) was 3.13. BSA, whose isoelectric point is 4.7,[30] is negatively charged in PBS (pH: 7.4). D-Octaarginine-linked PNVA-*co*-AA possessed an affinity to anionic FITC-BSA in neutral solution. Such interactions are commonly inhibited by SDS that strongly binds to proteins. SDS-PAGE also supported the interactions between the proteins and the polymers (Figure 2C).

This study demonstrated that there were electrostatic interactions between D-octaarginine-linked PNVA-*co*-AA and biomolecules with negative charges and that these biomolecules were taken up into cells without inactivation. Here, three issues remain unclear. The first is the requirement of electrostatic interactions for cellular uptake of biomolecules. Physicochemical properties of the interaction-based complexes, such as size and surface charge, are known to be critical for efficient in vitro transfection of nucleic acids that interact with cationic compounds.[31,32] Our results were in good agreement with conclusions of the previous report stating that positively charged complexes with a size of less than approximately 200 nm were efficiently taken up into cells.[33] The second issue is the fate of oligoarginine-linked polymers. Our previous research revealed that the majority of D-octaarginine-linked PNVA-*co*-AA remained on cell membranes.[23] Non- or little internalization of vehicles is advantageous to their safety. We also previously reported that the viability of cells incubated with D-octaarginine-linked PNVA-*co*-AA was as high as that observed under polymer-free conditions.[23] However, undoubted evidence for non-internalization of the polymers has not been obtained. We speculate that the polymers will be taken up into cells along with biomolecules if complex formation is a prerequisite for the cellular uptake of biomolecules. In such a case, it would be better to substitute PNVA-*co*-AA with biodegradable polymers because this biocompatible platform is not degraded in cells. On the other hand, observed activities of pGFP-C1 and β-galactosidase indicate that these biomolecules were free from polymers in cells. D-Octaarginine-linked PNVA-*co*-AA may be released from complexes before internalization unless complex formation is a prerequisite for the cellular uptake of biomolecules. The first and second issues will be discussed in a future report with the performance of polymers inside cells after internalization of biomolecules.

A mechanism for the cellular uptake of biomolecules incubated with oligoarginine-linked polymers is raised as the third issue. Suggestions of the mechanism can be obtained from the current research. As shown in Figure 4B, the MFI was constantly low when the mass concentration ratio of D-octaarginine-linked PNVA-*co*-AA to pGFP-C1 was 2.5 or less. When the concentration ratio exceeded 2.5, the MFI increased with a gradual elevation of the ratio, but physicochemical properties of complexes remained unchanged (Figure 3A). As mentioned above, the amount (mass) of polymers required for interactions with total pGFP-C1 was estimated to be 2.5 times that of the pDNA. It seemed that there were complexes and free polymers in solution where the concentration ratio was more than 2.5. Either full complexes or pGFP-C1 released from the complexes were possibly taken up into cells through macropinocytosis induced by free polymers. On the other hand, a different pattern was observed for FITC-BSA. Both complexes and free FITC-BSA were probably present when the mass concentration ratio of the polymer to FITC-BSA was less than 3.13 (Figure 2B). The MFI increased ratio-dependently under stable FITC-BSA concentration with different polymer concentrations even when free polymers were absent from the medium (Figure 6A). The electrostatic interaction of carboxyl groups in proteins is likely weaker than that of phosphoric acid esters in pDNA. It appeared that the replacement of counterparts, with which D-octaarginine in the polymer backbone interacted, occurred when complexes between FITC-BSA and polymers came in contact with cell membranes. D-Octaarginine-linked polymers that interacted with membrane-associated proteoglycans possibly enhanced membrane permeation of FITC-BSA released from the complexes. The MFI increase was probably saturated at a polymer concentration of 12.5 μg/mL, as the cell surface was entirely covered with polymers. Our speculation was also supported by the MFI obtained under stable polymer concentration with different FITC-BSA concentrations (Figure 6B). FITC-BSA concentration-dependent increase in the MFI was observed, and the highest value was obtained at the concentration ratio (polymer/FITC-BSA) of 2.5 even though it seemed that there was no free polymer. We suppose that oligoarginine-linked polymer-induced micropinocytosis results in the incidental uptake of insufficiently membrane permeable or membrane-impermeable molecules that are present in the periphery of the peptidyl branches. This strategy implies that the amount of molecules taken up into cells is proportional to the concentration of molecules applied to cells. Phenomena observed for FITC-BSA are consistent with our expectation.

Our cell-penetrating peptide-linked polymer is a potential candidate as an in vitro transfection tool that induces cellular uptake of biomolecules. It seems that the transfection mechanism differs from that of well-known transfection reagents such as lipofectamine, although further studies are required for its comprehension.

CONCLUSIONS

The potential use of D-octaarginine-linked PNVA-*co*-AA as an in vitro transfection tool for insufficiently membrane permeable or membrane-impermeable biomolecules was evaluated. Cell studies demonstrated that pGFP-C1 and β-galactosidase premixed with polymers were taken up into cells in their active forms. Complexes were formed through electrostatic interactions between anionic pGFP-C1 and cationic polymers. Cellular uptake of pGFP-C1 was initiated when complexes whose size and zeta potential were approximately 200 nm and 15 mV, respectively, were obtained. The uptake mechanism remained unclear; however, it seemed that free polymer-induced macropinocytosis resulted in the pDNA internalization. Electrostatic interaction-driven complex formation was also observed for BSA charged negatively in neutral solution. Due to frail interactions, membrane permeation of the proteins was possibly enhanced by not only free polymers, but also polymers released from complexes.

EXPERIMENTAL PROCEDURES

Materials. Sodium salts of PNVA-*co*-AA (catalog no.: GE-160-105, NVA units/AA units = 7:3, weight-average molecular weight (Mw): 350 kDa) were obtained from Showa Denko Co. (Tokyo, Japan). Octaarginine (D-configuration) with amidated terminal carboxyl groups was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). Other chemicals were commercial products of analytical or reagent grade and were used without further purification.

pGFP-C1 was obtained from Clontech Laboratories (Mountain View, CA). The pDNA was amplified in the

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JM109 strain of *Escherichia coli* and purified using a NucleoBond Xxtra Midi Kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany). FITC-BSA was purchased from Life Technologies (Rockville, MD). β-Galactosidase and β-Galactosidase Staining Kit were obtained from Promega Co. (Madison, WI) and Clontech Laboratories, respectively. Thermo Scientific M-PER Reagent was purchased from Thermo Scientific (Tokyo, Japan). Dulbecco's phosphate-buffered saline, modified (PBS without calcium chloride and magnesium chloride), was obtained from Sigma-Aldrich (St. Louis, MO).

HeLa and CHO cells were furnished by the American Type Culture Collection (Rockville, MD) and DS Pharma Biomedical Co., Ltd. (Osaka, Japan), respectively. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Ham's F-12 was obtained from DS Pharma Biomedical Co., Ltd. Phenol red-free DMEM, Opti-modified Eagle's medium (Opti-MEM), heat-inactivated fetal bovine serum (FBS), antibiotics (penicillin: 10 000 units/mL, streptomycin: 10 mg/mL), and trypsin-EDTA (0.25% trypsin and 1 mM EDTA) were purchased from GIBCO Laboratories (Lenexa, KS). L-Glutamine (200 mM) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Synthesis of D-Octaarginine-Linked PNVA-*co*-AA. D-Octaarginine-linked polymers were prepared in the same manner as previously described.[22−25] Briefly, sodium ions-free PNVA-*co*-AA was dissolved in *N*,*N*′-dimethylformamide (DMF) containing *N*,*N*′-dicyclohexylcarbodiimide (DCC). *N*-Hydroxysuccinimide (HOSu) was reacted with the DCC-activated carboxyl groups of PNVA-*co*-AA. The resulting PNVA-*co*-AA *N*-hydroxysuccinimide ester (PNVA-*co*-AA-OSu) was collected through reprecipitation in acetonitrile. PNVA-*co*-AA-OSu and D-octaarginine were dissolved in DMF. The terminal amino groups of D-octaarginine were coupled to the carboxyl groups of PNVA-*co*-AA through replacement of the *N*-oxysuccinimide groups. The resulting D-octaarginine-linked PNVA-*co*-AA was reprecipitated in acetonitrile, dialyzed in purified water, and lyophilized. The degree of D-octaarginine grafted onto PNVA-*co*-AA was measured by proton-NMR and expressed as the percentage of monomer units of acrylic acid (AA) grafting D-octaarginine to the total number of monomer units. D-Octaarginine-linked PNVA-*co*-AA with a grafting degree of 15%, whose performance was thoroughly examined in our previous study,[25] was used in this study. Weight percentage of D-octaarginine grafted onto PNVA-*co*-AA and Mw of D-octaarginine-linked polymers were calculated to be 74% and 1160 kDa, respectively.

Interactions between Biomolecules and D-Octaarginine-Linked PNVA-*co*-AA. *Electrophoresis.* Agarose gel electrophoresis was performed for pGFP-C1. The pDNA was mixed with D-octaarginine-linked PNVA-*co*-AA in PBS at a predetermined mass concentration ratio (pDNA concentration: 100 μg/mL). The mixture was then left for 15 min at room temperature. Each sample (10 μL) was loaded onto an 0.8% agarose gel at a pDNA amount of 1 μg. Electrophoresis was carried out at 100 V for 15 min in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and subsequently observed using a GelDoc XR System with Quantity One 1-D analysis software (Bio-Rad Laboratories, Tokyo, Japan).

Native-PAGE and SDS-PAGE were performed for FITC-BSA. A gel for native-PAGE was prepared using a 4% stacking gel and a 12% running gel. FITC-BSA was mixed with D-octaarginine-linked PNVA-*co*-AA in PBS at a predetermined mass concentration ratio (FITC-BSA concentration: 5 μg/mL). The mixture was then left for 15 min at room temperature. Each sample (10 μL) was loaded onto the gel at a FITC-BSA amount of 0.05 μg. Electrophoresis was carried out at 200 V for 60 min in Tris-borate-EDTA buffer. The unstained gel was observed using a ChemiDoc MP imaging System with Image Lab software (Bio-Rad Laboratories). SDS-PAGE was performed in the same manner as described above, except that SDS was dissolved in gels, samples, and buffer at a concentration of 0.1% (w/v).

Dynamic Light Scattering Spectrophotometry. Either pGFP-C1 or FITC-free BSA was mixed with D-octaarginine-linked PNVA-*co*-AA in PBS at a predetermined mass concentration ratio (concentrations of pGFP-C1 and BSA: 10 μg/mL and 20 μg/mL). The mixture was then left for 15 min at room temperature. The size of particles formed in PBS was measured by dynamic light scattering spectrophotometry (ELS-Z2, Otsuka Electronics Co., Osaka, Japan). Measurement time and temperature were set to 180 s (the measurement was repeated 70 times) and 25 °C, respectively. The zeta potential of the particles was also measured by electrophoretic light scattering spectrophotometry (ELS-Z2) at 25 °C.

Cellular Uptake of Biomolecules Mixed with D-Octaarginine-Linked PNVA-*co*-AA. *Cell Culture.* HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37 °C in humidified air containing 5% CO_2. CHO cells were cultured in Ham's F-12 supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37 °C in humidified air containing 5% CO_2.

Fluorescence Microphotography. HeLa cells were incubated at 37 °C for 24 h after they were seeded at a density of 1.0×10^4 cells/0.1 mL in each well of a 96-well plate. A mixture of pGFP-C1 (5 μg/mL) and D-octaarginine-linked PNVA-*co*-AA (25 μg/mL) was prepared using Opti-MEM as a solvent and then left for 15 min at room temperature. The culture medium was replaced with an equivalent volume of either the mixture or Opti-MEM containing the pDNA alone. After a 4 h incubation at 37 °C, the medium was removed and cells were washed with PBS twice. Cells were subsequently incubated in the culture medium at 37 °C for 68 h. After the medium was substituted with phenol red-free DMEM, fluorescence microphotographs of cells were obtained using a fluorescence microscope (magnification: ×100; excitation: 470−495 nm; emission: 510−550 nm; exposure: 1/5 of a second; Olympus IX71, Olympus Optical Co. Ltd., Tokyo, Japan). Optical microphotographs were also obtained at the same position where the fluorescence microphotographs were taken (magnification: ×100; exposure: 1/300 of a second).

HeLa cells immobilized on the 96-well plate were prepared in the same manner as described above. The culture medium was replaced with an equivalent volume of Opti-MEM containing FITC-BSA (5 μg/mL) with or without D-octaarginine-linked PNVA-*co*-AA (12.5 μg/mL) which was prepared in a manner similar to that described above. Temperature was set to either 37 or 4 °C. After a 1 h incubation, the medium was removed and cells were washed with PBS twice. Fluorescence and optical microphotographs of cells immersed in phenol red-free DMEM were obtained in the same manner as described above, except that exposure for the fluorescence image was set to 1/20 of a second.

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Flow Cytometry. HeLa cells were incubated with either pGFP-C1 or FITC-BSA by means of the same procedure as described in the previous section, with exception of following conditions: cells were seeded at a density of 1.0×10^5 cells/0.5 mL in each well of a 24-well plate; concentrations of pGFP-C1, FITC-BSA, and D-octaarginine-linked PNVA-*co*-AA were adjusted to 2 μg/mL, 0.5−5 μg/mL, and 0−25 μg/mL, respectively. Instead of phenol red-free DMEM immersion, cells were treated with 0.2 mL of trypsin-EDTA within a few minutes. The MFI of the harvested cells was measured using either fluorescence-activated cell sorter (Cyto ACE-300, JASCO Co., Tokyo, Japan; Guava easyCyte 6HT-2L, Merck KGaA, Darmstadt, Germany). Excitation wavelength was set to 488 nm. Emission wavelength was set to 530 nm for Cyto ACE-300 and 525 nm for Guava easyCyte 6HT-2L (green fluorescent channel of the cell sorter was used). The same test was performed for CHO cells.

Enzyme Assay. HeLa cells were incubated at 37 °C for 24 h after they were seeded at a density of 1.0×10^5 cells/0.5 mL in each well of a 24-well plate. β-Galactosidase (5 μg/mL) was mixed with D-octaarginine-linked PNVA-*co*-AA (0−25 μg/mL) in Opti-MEM and the mixture was then left for 15 min at room temperature. The cultured medium was replaced with an equivalent volume of the mixture. After a 1-h incubation at 37 °C, the medium was removed and cells were washed with PBS twice. Enzymatic activities of β-galactosidase taken up into cells were assayed using a β-Galactosidase Staining Kit. After cells were lysed with Thermo Scientific M-PER Reagent, the lysed solution were centrifuged at 20 000 g for 15 min at room temperature. The absorbance of the supernatant was measured at 415 nm using an iMark microplate reader (BioRad Laboratories).

Statistics. Statistical significance was assessed with the unpaired Student's *t* test, and *p* values of 0.05 or less were considered to be statistically significant.

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Notes

The authors declare no competing financial interest.

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ABBREBIATIONS

pGFP-C1, plasmid DNA encoding green fluorescent protein; BSA, bovine serum albumin; GFP, green fluorescent protein; PNVA-*co*-AA, poly(*N*-vinylacetamide-*co*-acrylic acid)

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