Synthesis and characterization of lactosylated poly(1-vinylimidazole) for cell-specific plasmid DNA carrier

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Abstract
Poly(1-vinylimidazole) (PVIm) with aminopropyl groups, that is, PVIm-Pr-NH₂, has been synthesized as an efficient plasmid DNA (pDNA) carrier. Then, the PVIm-Pr-NH₂ has been modified with lactose molecules as a cell-specific pDNA carrier. The resulting PVIm with lactose molecules, that is, PVIm-Pr-Lac, possessed approximately 12 mol% modified lactose units and formed the polyion complex (PIC) with pDNA at the positive/negative charge ratio of 2, 4, 8, or 16. The size of the resulting PVIm-Pr-Lac/pDNA PICs was within the range from approximately 100 nm to 200 nm. The PVIm-Pr-Lac/pDNA PICs specifically mediated the pDNA expression for human hepatoma HepG2 cells with asialoglycoprotein receptors. These results suggest that the PVIm-Pr-Lac worked as cell-specific pDNA carrier to deliver pDNA inside the cells for efficient gene expression.

Introduction
In the field of plasmid DNA (pDNA) delivery, the polyion complex (PIC) formation of between pDNA and polycation has widely been demonstrated as a new design of pDNA carrier [1, 2]. The pDNA carriers to deliver pDNA inside cells are internalized into acidic endosome, where the carriers are subjected to a pH change from pH 7 to 5 [3]. Therefore, the escape from the endosome is one of the important factors for the design of efficient pDNA carrier. Poly(ethyleneimine) (PEI) is one of pH-sensitive polycations with proton sponge effect to capture protons entering an endosome, resulting in the swelling of the endosomes to lead to membrane disruption for escape from endosomes [4].

On the other hand, the histidine-modified polycations or the polycations containing imidazole (Im) groups have enhanced pDNA expression [5-7]. In this case, the escape of the polycation/pDNA PIC from the endosome has been achieved by the proton sponge effect of Im groups including histidine. The pKa of the Im group is around 6, furthermore, the buffering capacity of Im groups around pH 6 in endosome induces the destabilization of cell membrane after their protonation. A similar effect is also observed with liposomes that include Im polar head [8]. The pH-sensitivity of the resulting Im groups in the pDNA carrier is considered to be critical for the release of the pDNA to cytosol. Based on these backgrounds, we have already reported several pDNA delivery systems based on Im groups [9-14]. Especially, we have already reported a poly(1-vinylimidazole) (PVIm) with several aminopropyl (Pr-NH₂) groups, that is, PVIm-Pr-NH₂, for a pH-sensitive polycation to enhance cell-specific pDNA delivery [14]. By using PVIm-Pr-NH₂ as a pH sensitive pDNA carrier, as well as a lactosylated poly(L-lysine) as a cell-targeting pDNA carrier, the resulting ternary complexes specifically mediate pDNA expression. pDNA expression depends on our original concept that pDNA ternary complexes dissociate ligand polycations in response to endosomal pH [14, 15]. However, PVIm-Pr-NH₂/pDNA binary complexes mediate no significant gene expression.

In this study, to develop PVIm-Et-NH₂ for the realization of efficient pDNA expression as well as alkylated PVIm (PVIm-R) [12], and to modify PVIm with lactose (Lac) covalently as a cell-targeting ligand, we have synthesized PVIm with several aminopropyl (Pr-NH₂) groups, that is, PVIm-Pr-NH₂. The resulting PVIm-Pr-NH₂ has been modified with lactose by the reductive amination between the amino group of PVIm-Pr-NH₂ and the reducing end of lactose. The final product of lactosylated PVIm, that is, PVIm-Pr-Lac, is expected to mediate the cell-specific efficient pDNA expression.

Materials and methods
Materials
3-Bromopropylamine hydrobromide and PEI solution were purchased from Sigma–Aldrich Co. LLC, St. Louis, MO, USA. All other chemicals of a special grade were used without further purification.

Synthesis of PVIm-Pr-Lac
The synthetic route of PVIm-Pr-Lac is shown in Scheme 1. Poly(1-vinylimidazole) (PVIm) was synthesized according to our previous paper [16]. The PVIm (60 mg) and 3-bromopropylamine (20 mg) were dissolved in 8 mL dimethyl sulfoxide (DMSO). The reaction mixture was incubated at 40°C for 2 days, followed by dialysis against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff=10⁶) to remove unreacted 3-bromopropylamine. The resulting polymer PVIm-Pr-NH₂ was obtained by freeze-drying.

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Key words: poly(1-vinylimidazole), lactose, plasmid DNA, cell-specific carrier, drug delivery system

Received: January 07, 2020; Accepted: January 20, 2020; Published: January 24, 2020
Then, the PVIm-Pr-NH₂ (20 mg) and lactose monohydrate (288 mg) were preincubated at 37°C for 3 days in 2.5 mL of sodium borate buffer (0.1 M, pH 8.5) [17]. Subsequently, a reducing agent, NaBH₄CN (193 mg), was added to the mixture of PVIm-Pr-NH₂ and lactose in 5 mL of the sodium borate buffer. The reaction mixture was further incubated at 37 °C for 3 days. The degree of amino group modification was examined by the primary amino group determination with fluorescamine [18]. The reaction mixture was dialyzed against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10⁵) to remove unreacted lactose, followed by freeze-drying.

### Agarose gel retardation assay

The resulting PVIm-Pr-Lac as well as PVIm-Pr-NH₂ and pDNA (pGL3-control vector; from Promega Co.) were mixed in PBS(-) at a positive/negative charge ratio of 2, 4, 8 or 16. After the incubation at 37 °C for 1 h, each sample (corresponding to 300 ng pDNA) was mixed with a loading buffer (BlueJuice™, Invitrogen/Life Technologies) and loaded onto a 1% agarose gel containing 1 μg/mL ethidium bromide (EtBr). Gel electrophoresis was run at room temperature in TAE buffer (Tris-acetate, EDTA) at 50 V for 30 min. The pDNA bands were visualized under UV irradiation.

### Size and ζ-potential measurement

The PVIm-Pr-Lac as well as PVIm-Pr-NH₂ and the pDNA were mixed in PBS(-) at a positive/negative charge ratio of 2, 4, 8 or 16. After the incubation at 37 °C for 2 h, the size of each sample was measured by a dynamic light scattering (DLS) method using an electrophoresis light scattering spectrophotometer (ELS-Z2, Otsuka Electronics Co., Ltd., Tokyo, Japan) and the zeta potential was measured by ELS with electrodes.

### Cell viability assay

HepG2 cells (from Riken Bioresource Center Cell Bank), human hepatoblastoma cell line, were cultured in tissue culture flasks containing Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated of fetal bovine serum (FBS). The cells were seeded at 1×10⁴ cells/well (100 µL/well) in a 96-well plate and incubated at 37°C in a 5% CO₂ incubator, overnight. After the addition of 15 µL PBS(-) containing the complex between 200 ng pDNA and PVIm-Pr-Lac as well as PVIm-Pr-NH₂ at a positive/negative charge ratio of 2, 4, 8 or 16, the cells were incubated at 37°C for 24 h. After changing the medium, the cells were further incubated for 48 h, followed by the Alamar Blue assay [19] in triplicate.

### Transfection procedure

According to the cell viability assay, 1×10⁴ cells/well HepG2 cells were transfected in DMEM supplemented with 10% heat-inactivated FBS by the addition of 15 µL PBS(-) containing 200 ng of each pDNA encoding the modified firefly luciferase and PVIm-Pr-Lac as well as PVIm-Pr-NH₂ at a positive/negative charge ratio of 2, 4, 8 or 16. PEI (positive/negative = 8) was used as a positive control. After 1 day of incubation, the medium was removed and the cells were further incubated for 2 days in the DMEM supplemented with 10% FBS. After washing the cells with PBS(-) twice, the cells were assayed by the luciferase protocol (Promega kit), according to the manufacturer’s instructions. Luciferase activities were normalized to protein concentrations and the results were presented as relative light units (RLU). Protein concentrations were determined by the bicinchonic acid (BCA) protein assay kit (Pierce), according to the manufacturer's instructions.

### Results and discussion

#### Synthesis of PVIm-Pr-Lac as well as PVIm-Pr-NH₂

As shown in Scheme 1, PVIm was reacted with 3-bromopropylamine for alkylation to obtain primary amino groups as well as quaternary imidazole groups. The amino groups of the resulting PVIm-Pr-NH₂ were subsequently reacted with the reducing end of lactose by reductive amination to obtain alkylated PVIm with lactose, PVIm-Pr-Lac. As shown in Figure 1, the ¹H NMR spectrum showed the characteristic signals of PVIm backbone (Figure 1) and propylene. From the signal ratio, the modification ratio of aminopropylated imidazole groups was calculated to be 12 mol%. Furthermore, as shown in Figure 1, the ¹H NMR spectrum showed the characteristic signals of PVIm-Pr-NH₂ backbone and lactose. By the primary amino group determination with fluorescamine (results not shown), as well as ¹H NMR signals, only 5 mol% amino groups were remained after the reductive amination reaction, suggesting the almost complete modification (95 mol%) of the PVIm-Pr-NH₂ with lactose. Thus, we have synthesized the lactosylated PVIm, that is, PVIm-Pr-Lac, as well as the PVIm with several aminopropyl groups, that is, PVIm-Pr-NH₂.

#### PIC formation between pDNA and PVIm-Pr-Lac as well as PVIm-Pr-NH₂

As shown in Figure 2, we examined whether the PVIm-Pr-Lac as well as PVIm-Pr-NH₂ formed the PIC with pDNA by agarose gel electrophoresis. The complete retardation of pDNA in the agarose gel proved that the PVIm-Pr-Lac as well as PVIm-Pr-NH₂ formed the PIC.
with pDNA above positive/negative charge ratio of 2. Because of the coil-globule transition of pDNA and the resulting inhibition of the EtBr intercalation [20,21], no fluorescence owing to EtBr staining was observed in case of the PEI/pDNA PIC at the positive/negative charge ratio of 8. On the other hand, slight fluorescence was observed in case of the PVIm-Pr-NH$_2$/pDNA PICs at the positive/negative charge ratio of 8. The nonionic PVIm backbone is considered to solubilize the pDNA PICs and to reduce the coil-globule transition of pDNA, that is, pDNA compaction, at the charge ratio [14,22]. Furthermore, a significant fluorescence was observed in case of the PVIm-Pr-Lac/pDNA PICs. A similar observation was previously reported in the case of the soluble complexes between pDNA and the polycation grafted with water-soluble carbohydrate chains such as dextran [23,24]. In this study, the water-soluble carbohydrate lactose seems to pDNA PICs and to reduce pDNA compaction.

Accordingly, we measure the particle size and ζ-potential of the PVIm-Pr-Lac/pDNA PICs (Table 1) as well as the PVIm-Pr-NH$_2$/pDNA PICs (Table 2). The particle size of the PVIm-Pr-Lac/pDNA PICs is almost similar to that of the PVIm-Pr-NH$_2$/pDNA PICs in the rage around 200 nm. On the other hand, the ζ-potential of PVIm-Pr-Lac/pDNA PICs seems to be a little lower than that of the PVIm-Pr-NH$_2$/pDNA PICs, suggesting the slight shielding of surface positive charges by nonionic carbohydrate lactose.

Cell-Specific pDNA delivery by PVIm-Pr-Lac/pDNA PICs

To confirm the required properties as pDNA carrier, as shown in Figure 3, we first carried out a cytotoxicity assay. The cell viability of HepG2 hepatoma cells kept over 90% in the presence of the PVIm-Pr-Lac/pDNA PIC at the positive/negative charge ratio of 16. On the other hand, the cell viability decreased up to 80% in the presence of the PVIm-Pr-NH$_2$/pDNA PIC at the positive/negative charge ratio of 16, when the cell viability decreased up to 60% in the presence of the PEI/pDNA PIC as a control even at the positive/negative charge ratio of 8. These results suggest that the highest positive ζ-potential of the the PVIm-Pr-NH$_2$/pDNA PIC at the positive/negative charge ratio of 16 (Tables 1 and 2) damaged a negative-charged cell membrane. Therefore, the PVIm-Pr-Lac with no apparent cytotoxicity promises to be an effective pDNA carrier.

As a no apparent cytotoxicity, we finally examined the pDNA expression mediated by the PVIm-Pr-Lac/pDNA PICs as well as PVIm-
Pr-NH₂/pDNA PICs. HepG2 cells are a human hepatoma cell line, so that asialoglycoprotein receptor (ASGP-R) expressed on the cell surface [16]. As shown in Figure 4, the PVIm-Pr-Lac/pDNA PICs mediated remarkable pDNA expression, which was almost the same level as that mediated by the control PEI, on HepG2 cells. On the other hand, the pDNA expression mediated by the PVIm-Pr-NH₂/pDNA PICs was lower than that mediated by the PVIm-Pr-Lac/pDNA PICs. On human cervical carcinoma (HeLa) cell line with no ASGP-R on the cell surface, as shown in Figure 4, almost no difference between PVIm-Pr-Lac and PVIm-Pr-NH₂ was observed. Namely, the pDNA expression mediated by the PVIm-Pr-Lac/pDNA PICs was almost the same level as that mediated by the PVIm-Pr-NH₂/pDNA PICs on HeLa cells. These results suggest that the PVIm-Pr-Lac/pDNA PICs with β-galactose residues recognized the HepG2 cells via ASGP-Rs on the cell surface.

Conclusion

We have synthesized the PVIm-Pr-NH₂ and PVIm-Pr-Lac as derivatives from our pH-sensitive pDNA carrier PVIm-R. Especially, the PVIm-Pr-Lac possessed approximately 12 mol% modified lactose units and formed the PICs with pDNA. The resulting PVIm-Pr-Lac/pDNA PICs specifically mediated the pDNA expression for human hepatoma with asialoglycoprotein receptors. These results suggest that the PVIm-Pr-Lac worked as cell-specific pDNA carrier to deliver pDNA inside the cells for efficient gene expression.

Acknowledgements

This research was partially supported by a Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science (JSPS KAKENHI grant no. 16H03183).
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