Plasmid DNA Mono-Ion Complex for in Vivo Sustainable Gene Expression

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Supporting Information

ABSTRACT: To cleave biocompatible poly(ethylene glycol) (PEG) from the mono-ion complex (MIC) for sustainable cellular uptake in vivo, ω-amide-pentylimidazolium end-modified PEG with an ester bond, that is, APe-Im-E-PEG, has been synthesized. The hydrolysis of the resulting APe-Im-E-PEG proceeded during the incubation for 2 weeks under physiological conditions, which was confirmed by gel filtration chromatography. APe-Im-E-PEG formed the MIC with plasmid DNA (pDNA), assessed by agarose gel retardation assay. Furthermore, dynamic light scattering measurement and transmission electron microscopy observations have estimated that the particle size of the resulting MIC was approximately 30 nm, with a rather flexible structure. The APe-Im-E-PEG/pDNA MIC incubated for 2 weeks exhibited hemolytic activity at endosomal pH, presumably because the pH-sensitive carboxyl groups revealed after the hydrolysis of an ester bond of APe-Im-E-PEG. The APe-Im-E-PEG/pDNA MIC enhanced the gene expression 2 weeks after transfection in vivo by intramuscular administration in mice. Consequently, in vivo sustainable gene expression has been achieved by the molecular design of APe-Im-E-PEG for cellular uptake and endosomal escape proceeded by temporal hydrolysis of the ester bond.

INTRODUCTION

Plasmid DNAs (pDNAs) have the advantage to produce many therapeutic proteins by translation as well as many therapeutic RNAs by transcription. For clinical application, the gene expression from pDNA should be preserved in vivo, resulting in sustainable production of therapeutic proteins as well as RNAs. Accordingly, the strategy design for in vivo sustainable gene expression is considered to be important target in the field of pDNA delivery to disease site of bedside patients. In the field of pDNA delivery system, various polycationic complexes (PICs) between pDNA and polycations by electrostatic interaction are widely used for pDNA transfection in vitro and in vivo. Especially, in vivo pDNA delivery systems incorporate poly(ethylene glycol) (PEG) to enhance the biocompatibility of PICs, such as retention in a living body for sustainable protection from PEG degradation in vivo.

Recently, to overcome the structural properties of PICs, such as a tendency for large particle size by cross-linking between polycations and pDNAs, we have reported the concept of mono-ion complex (MIC) between our original monocationic PEG and pDNA. As a stabilized MIC by hydrogen bond with an adenine base in adenine–thymine base pair of pDNA, we have already designed ω-amide-pentylimidazolium end-modified PEG, i.e., APe-Im-PEG.

Including our MIC, although the incorporation of PEG enhances the biocompatibility to suppress nonspecific interaction with mainly serum proteins, the resulting PEG shielding often blocks the accessibility to target cells. The trade-off problem is known as PEG dilemma. Accordingly, various strategies for overcoming the PEG dilemma are reported such as cleavage of PEG from the delivery system. The cleavable PEGylation is triggered by several kinds of mechanisms such as the acidolysis of a hydrazine group in the hydrolysis of an ester bond in extracellular microenvironment, or the thiolysis of a disulfide bond in intracellular cytoplasm.

In this study, to overcome the PEG dilemma to access target cells in spite of PEG protection for in vivo sustainable gene expression, we have designed ω-amide-pentylimidazolium end-modified PEG with an ester bond to be gradually hydrolyzed for sustainable cellular uptake, i.e., APe-Im-E-PEG (Figure 1). Furthermore, the hydrolyze of an ester bond in this study is designed to produce a pH-sensitive carboxyl group on the side of the APe-Im-E-PEG/pDNA MIC for endosomal escape.

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The resulting de-PEGylated MIC with pH sensitivity is therefore expected to achieve sustainable gene expression from pDNA in vivo.

RESULTS AND DISCUSSION

Synthesis and Stability of APe-Im-E-PEG. Figure 1 shows the synthesis scheme of APe-Im-E-PEG to form the MIC with pDNA for in vivo sustainable gene expression. To resolve the PEG dilemma, we have introduced an ester bond between ω-amide-pentylimidazolium (APe-Im) and PEG. The hydrolysis of the ester bond is expected to dissociate PEG from the MIC for sustainable cellular uptake. To proceed to endosomal escape, furthermore, we have adjusted the direction of the ester bond to produce a pH-sensitive carboxyl group on the side of the MIC after the hydrolysis. The resulting carboxyl group is expected to disturb the endosomal membrane under acidic pH conditions inside the endosome for the promotion of cytoplasmic delivery.

The synthesis scheme of APe-Im-E-PEG is shown in Figure 2. First, we reacted α-succinimidoylsuccinyl-ω-methoxy, polyeoxyethylene (NHS-E-PEG) with 1-(3-aminopropyl)imidazole, resulting in imidazole end-modified PEG (Im-E-PEG). Im-E-PEG was subsequently reacted with 6-bromohexaneamide for alkylation to synthesize a quaternary imidazole group of PEG. Final product yield was approximately 25%. The 1H NMR spectrum indicated the signal of a ω-amide-pentyl group (APe), a propylene imidazolium group (Im), and a succinyl PEG (E-PEG) (see Figure S1 in the Supporting Information). From the suitable signal ratio, we have successfully synthesized ω-amide-pentylimidazolium end-modified PEG with an ester bond, i.e., APe-Im-E-PEG.

Hydrolysis Properties of APe-Im-E-PEG. Figure 3 shows whether APe-Im-E-PEG was hydrolyzed under physiological (pH 7.4) or acidic (pH 5.0) conditions by gel filtration chromatography (GFC). During the incubation for 2 weeks at pH 7.4, the retention time (RT) of imidazolium detected by absorbance (ABS) at 300 nm was gradually delayed; namely, the peak at RT of 9.95 min was gradually increased compared with that at RT of 9.00 min (Figure 3a). Conversely, during incubation at pH 5.0, the imidazolium peak ratio of RT of 9.95 min to RT of 9.00 min was almost constant (Figure 3b). Furthermore, the peak at RT of 9.53 min was detected by refractive index (RI), which was not observed by the detection of ABS at 300 nm, was gradually increased compared with that at RT of 9.00 during incubation for 2 weeks at pH 7.4 (Figure 3c and see Figure S2 in the Supporting Information). From these results, APe-Im-E-PEG is considered to be gradually hydrolyzed under physiological pH conditions to produce an imidazolium moiety with ABS at 300 nm and a PEG without ABS.

pH-Dependent Hemolytic Activity of APe-Im-E-PEG. To examine the pH-dependent hemolytic activity of the produced carboxyl groups after the hydrolysis of APe-Im-E-PEG, we first examined the native cytotoxicity of APe-Im-E-PEG before hydrolysis (Figure 4a) because the cytotoxicity of
APe-Im-E-PEG is an important factor for clinical applications. The overall cytotoxicity of free APe-Im-E-PEG and polycations is higher than that of the corresponding complexes. Therefore, we choose the cytotoxicity assay of the free APe-Im-E-PEG and polycations to give a worst-case estimate of the interaction of APe-Im-E-PEG and polycations with cells rather than that of the MIC and PICs with pDNA. APe-Im-E-PEG maintained almost 100% cell viability after one day incubation with human cell line (HepG2) as a representative human cell for future clinical application in spite of the existence of a cationic group in the polymer. Conversely, poly(L-lysine) (PLL) and branched poly(ethylenimine) (b-PEI) with primary ammonium groups decreased cell viability in view of the same molar concentration of cationic groups. These results are consistent with our previous study that dimethyl imidazolium groups were noncytotoxic species as compared to PLL and b-PEI.39 Because of a kind of imidazolium group, therefore, the ω-amide-pentylimidazolium is considered to be a noncytotoxic cation.

Then, the hemolytic activity was examined by mixing erythrocytes and APe-Im-E-PEG, incubated for 1 week or 2 weeks under physiological conditions, in the isotonic buffer at pH 7.4 or pH 5.0 (Figure 4b). At pH 7.4, no increase in the absorbance at 577 nm, from hemoglobin release, occurred when the erythrocytes were mixed with APe-Im-E-PEG incubated for 2 weeks. On the other hand, hemoglobin release was significantly increased at pH 5.0 (see Figure S3 in the Supporting Information) as the incubation time for hydrolysis of APe-Im-E-PEG increased from 0 day to 2 weeks. These results suggest that the pH-dependent carboxyl groups were produced after the hydrolysis of APe-Im-E-PEG to induce the hemolytic activity by the protonated carboxyl groups at pH 5.0. The resulting hemolytic activity is considered to promote endosomal escape by the cell (endosome) membrane disruption inside late endosome at pH 5.0.40

Figure 4. (a) Effect of APe-Im-E-PEG on cell viability: (●) APe-Im-E-PEG; (▲) PLL; and (⧫) b-PEI. Symbols and error bars represent the mean and standard deviation of the measurements made in triplicate wells. (b) Effect of pH on the hemolytic activity of APe-Im-E-PEG incubated for 1 week or 2 weeks at 37 °C: pH 7.4 (gray bars) or pH 5.0 (black bars). The released hemoglobin (Hb) was determined by measuring the absorbance at 577 nm.
Formation of the pDNA MIC with APe-Im-E-PEG. To prove the MIC formation of the resulting APe-Im-E-PEG with pDNA, we carried out agarose gel electrophoresis (Figure 5a).

The migration of supercoiled pDNA band was almost completely retarded above the positive/negative charge ratio of 16 or 32, not depending on the concentration of dextran sulfate. From these results, in spite of monovalent ionic interaction, APe-Im-E-PEG/pDNA MIC at higher mixing charge ratios is considered to be stable against competitive exchange with polyanion by multivalent ionic interaction. Furthermore, to check the stability of the APe-Im-E-PEG/pDNA MIC in serum protein, we carried out the agarose gel electrophoresis of the MIC in the presence of fetal bovine serum (FBS) (see Figure S5 in the Supporting Information). The MIC bands were retained at the mixing charge ratio of 16 or 32, suggesting negligible reaction of the ester bond with the amino groups of protein.

From the viewpoint of overcoming the PEG dilemma, to monitor the size and the surface charge, as well as pDNA condensation degree before and after hydrolysis, we carried out the agarose gel electrophoresis of the APe-Im-E-PEG/pDNA MIC incubated for 2 weeks (see Figure S6 in the Supporting Information). As the incubation time increased up to 2 weeks, the MIC bands at the mixing charge ratio of 16 or 32 were further migrated and not apparently stained by ethidium bromide (EtBr). These results suggest that the appearance of the anionic carboxyl group on the side of the MIC after hydrolysis promoted the further migration into the plus pole of the gel. Also, the cleavage of expanded PEG is considered to induce the coil–globule transition of pDNA, resulting in the inhibition of the EtBr intercalation.11

In Vivo Sustainable Gene Expression Mediated by the APe-Im-E-PEG/pDNA MIC. According to the stability of the APe-Im-E-PEG/pDNA MIC, we finally checked whether MIC mediated the sustainable gene expression. As shown in Figure 7, we determined the gene expression from the APe-Im-E-PEG/pDNA MIC injected into the skeletal muscle after 1 week or 2 weeks. In case of intramuscular administration, naked pDNAs are used for some clinical trials to mediate significant gene expression.41–43 The gene expression mediated...
by naked pDNA lowered up to 25% from 1 week to 2 week after transfection. As compared to naked pDNA 1 week after transfection, approximately 5 times higher gene expression was obtained by the MIC at the mixing charge ratio of 32. However, after 2 weeks, the resulting gene expression reduced up to 20% level from 1 week after transfection. At the mixing charge ratio of 16, the gene expression mediated by the MIC was 1.5 times higher than that mediated by naked pDNA 1 week after transfection. At 2 week post-transfection, notably, the gene expression mediated by MIC was 8.5 times higher than that mediated by naked pDNA (p < 0.05). Namely, it is worth noting that the resulting gene expression rose by 50% from 1 week to 2 weeks after transfection. These results suggest that APe-Im-E-PEG/pDNA MIC at the mixing charge ratio of 16 was an optimum structure for in vivo sustainable gene expression under these experimental conditions. The reason why the mixing charge ratio of 16 showed a more sustainable gene expression may be homogeneity of the MIC with small particle size below 30 nm, in spite of no significant difference of morphology in the TEM images, because the MIC with large particle size may be partially formed by accidental aggregation due to a large number of APe-Im-E-PEG at the mixing charge ratio of 16. Collectively, although details of the mechanisms are now under investigation, APe-Im-E-PEG is considered to be a suitable design for a chemical structure with an ester bond between the APe-Im group and the PEG chain to sustain the gene expression in vivo.

![Figure 7. Transfection activity of luciferase gene into the skeletal muscles injected by the APe-Im-E-PEG/pDNA MIC at [ω-amidepentylimidazolium][APe-Im-E-PEG]/[phosphate]pDNA ratios (charge ratio: +/-) of 1–32 after 1 week (gray bars) or 2 weeks (black bars). Gene expression was determined as relative light unit (RLU) normalized by the protein concentration. Symbols and error bars represent the mean and standard deviation. Statistical significance (p < 0.05) is indicated when compared to the naked pDNA (n = 6).](image)

**CONCLUSIONS**

In vivo sustainable gene expression 2 weeks after transfection has been achieved by our original concept of the MIC between pDNA and APe-Im-E-PEG. APe-Im-E-PEG is designed to cleave PEG from the MIC gradually for 2 weeks, resulting in the hemolytic activity under acidic pH conditions for the endosomal escape to enhance gene expression. The achievement of in vivo sustainable gene expression for 2 weeks by our pDNA MIC is promising for clinical application to produce therapeutic proteins continuously using pDNA.

**EXPERIMENTAL PROCEDURES**

**Materials.** α-Succinimidyloxy succinyl-ω-methoxy, polyoxyethylene (NHS-PEG) (average molecular weight of 2267) was purchased from NOF corporation (Tokyo, Japan). 1-(3-Aminopropyl)imidazole was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 6-Bromohexamamide was purchased from Combi-Blocks Inc. (San Diego, CA). All other chemicals were of special grade and used without further purification.

**Synthesis of APe-Im-E-PEG.** A typical procedure is as follows (Figure 1): NHS-PEG (400 mg, 0.2 mmol) and 1-(3-aminopropyl)imidazole (118 μL, 1.0 mmol) were mixed in 3 mL of N,N-dimethylformamide (DMF) in the presence of 139 μL (1.0 mmol) of triethylamine (TEA) and incubated at 50 °C for 24 h. The resulting mixture was dialyzed against distilled water with the Spectra/Por CE membrane (molecular weight cutoff of 100–500), followed by freeze-drying.

The resulting sample (Im-E-PEG) (240 mg: 0.1 mmol) and 6-bromohexamamide (194 mg: 1 mmol) were mixed in 10 mL of DMF in the presence of 14 μL (0.1 mmol) of TEA and incubated at 50 °C for 24 h. To the resulting mixture, 6-bromohexamamide (194 mg: 1 mmol) was repeatedly added. The resulting mixture was further incubated at 50 °C for 24 h. Then, the resulting mixture was dialyzed against distilled water with the Spectra/Por CE membrane (molecular weight cutoff of 100–500). The resulting sample (APe-Im-E-PEG) after the dialysis was collected by freeze-drying.

**1H NMR Spectroscopy.** The polymer (5 mg) was dissolved in 600 μL of D2O (99.8 atom % deuterium; Acros, NJ). The 1H NMR spectra (500 MHz) were recorded by a Bruker AV500 spectrometer (Billerica, MA).

**Gel Filtration Chromatography (GFC).** GFC was carried out using a JASCO PU-980 pumping system (Tokyo, Japan) at the flow rate of 1.0 mL/min with a Shodex OHpak SB-804 HQ column (Showa Denko K. K., Tokyo, Japan). The aqueous solution containing PBS(-) was used as a mobile phase. One hundred microliters of 1 mg/mL samples, which were incubated at 37 °C for 2 days in 4-(2-hydroxyethyl)1-piperazinethanesulfonic acid) buffer at pH 7.4 or pH 5.0, were injected into the column. The eluate was detected with a RI detector (RI-1530; Jasco) and a UV detector (UV-2077; Jasco).

**Agarose Gel Retardation Assay.** A stock solution of APe-Im-E-PEG (0.5–7.9 μL) was added to the dilution (500 ng of pDNA) of pDNA stock solution with H2O. Then, the final volume was adjusted to 13.5 μL at various [ω-amidepentylimidazolium][APe-Im-E-PEG]/[phosphate]pDNA ratios. The resulting mixture was incubated at 37 °C for 24 h, and mixed with a loading buffer (1.5 μL). Subsequently, the resulting sample was loaded onto a 1% agarose gel containing 1 μg/mL EtBr. Gel electrophoresis (50 V, 30 min) was carried out at room temperature in TAE buffer (Tris-acetate, ethylenediaminetetraacetic acid (EDTA)). pDNA was electrophoresed with size standard maker, and the pDNA bands were visualized under UV irradiation. For the competitive inhibition of the MIC formation, gel electrophoresis was run in the presence of dextran sulfate (1–4 mM as sulfate group) incubated at 37 °C for 2 h after the MIC formation. For the competitive exchange of MIC, furthermore, the electrophoresis was carried out in the presence of 10% FBS incubated at 37 °C for 5 min after the MIC formation.
Particle Size Measurement. The size of pDNA (13.3 μg) incubated at 37 °C for 24 h with APe-Im-E-PEG at various [ω-amide-pentylimidazolidum]APe-Im-E-PEG/[phosphate]pDNA ratios was measured in 100 μL of phosphate-buffered saline without divalent cations, i.e., PBS(−), by a dynamic light scattering method using an electrophoresis light scattering spectrophotometer (ELS-Z2, Otsuka Electronics Co., Ltd., Tokyo, Japan) and the ζ-potential was measured by ELS with electrodes.

Transmission Electron Microscopy (TEM) Observations of the MIC Structures. A TEM sample solution for observing the MIC was prepared by mixing 10 μL of a twice-diluted MIC solution with 10 μL of 2% uranyl acetate on ice. A TEM grid (Nissin EM Co., Tokyo, Japan), which had been hydrophilized by an Eiko IB-3 ion coater (Eiko Engineering Co., Ltd., Shimane, Japan), was dipped into the sample solution for 45 s. The excess solution was blotted away. The grids were observed by a JEM-1400 Bio-TEM (JEOL Ltd., Tokyo, Japan) operated at an acceleration voltage of 120 kV.

Cell Viability Assay. HepG2 cells (from Riken Biobank Center Cell Bank), human hepatoma cell line as a representative cell, were cultured in tissue culture flasks containing Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS. The cells were seeded at 1 × 10⁴ cells/well in a 96-well plate and incubated overnight at 37 °C in a 5% CO₂ incubator. The cells were treated with APe-Im-E-PEG (0–10 mg/mL) and incubated for 24 h at 37 °C. By additional incubation for 4 h, the cell viability was measured using the Alamar Blue assay²⁴ in triplicate.

Hemolysis Assay. Eighty microliters of the stock solution (10 mg/mL) of APe-Im-E-PEG was incubated for 1 or 2 weeks at 37 °C. Then, partial 50 μL of the stock solution was added to 1.25 mL of the 10 mM sodium phosphate buffer (pH 7.4 and pH 5.0) containing 130 mM NaCl. Then, 1.3 mL of each APe-Im-E-PEG solution was incubated with 100 μL of rabbit erythrocytes (kindly given by Keio University, Tokyo, Japan) for 1 h at 37 °C. After centrifugation (13 000 rpm, 10 min, 4 °C), the released hemoglobin (Hb) was determined by measuring the absorbance at 577 nm of the 3-fold diluted supernatant.

In Vivo Gene Delivery into the Skeletal Muscles by APe-Im-E-PEG. In vivo gene delivery into the skeletal muscles of mice with APe-Im-E-PEG was carried out using previously described methods.²⁵ Briefly, ICR mice (5 weeks old, male) were anesthetized with pentobarbital. The complexes between pDNA (5 μg) and APe-Im-E-PEG at various [ω-amide-pentylimidazolidum]APe-Im-E-PEG/[phosphate]pDNA ratios in 35 μL of PBS(−), which were incubated at 37 °C for 24 h, were injected into the tibialis muscles of the ICR mice. One week or two weeks after the injection, the whole tibialis muscles were injected with 1.25 mL of the 10 mM sodium phosphate buffer for 45 s. The excess solution was blotted away. The grids were observed by a JEM-1400 Bio-TEM (JEOL Ltd., Tokyo, Japan) operated at an acceleration voltage of 120 kV.

Statistical Analysis. Statistical analysis was performed using the two sample equal variance Student’s t-test.

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