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Enhanced stability and gene silencing ability of siRNA-loaded polyion complexes formulated from polyaspartamide derivatives with a repetitive array of amino groups in the side chain

Tomoya Suma [a], Kanjiro Miyata [b], Takehiko Ishii [a], Satoshi Uchida [b], Hirokuni Uchida [a], Keiji Itaka [b], Nobuhiro Nishiyama [b], Kazunori Kataoka [a,b,c,d,*]

[a] Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[b] Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[d] Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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ABSTRACT

The delivery of siRNA therapeutics owes its success to the development of carrier systems with high efficacy and minimum toxicity. Here, cationic polyaspartamide derivatives with a regulated number and spacing of positively charged amino groups in the side chain were prepared from a single platform polymer of poly(β-benzyl L-aspartate) to assess their availability as siRNA carriers through polyion complex (PIC) formation. These polymers have 1,2-diaminoethane, 1,3-diaminopropane, and *N,N'*-bis(2-aminoethyl)-1,2-diaminoethane moieties in the side chain, and are termed as PAsp(DET), PAsp(DPT), and PAsp(TEP), respectively. siRNA-loaded PICs stable in serum-containing media were formed from PAsp(TEP) and PAsp(DPT) with two positive charges in the side chain at pH 7.4, whereas no such stable PIC was obtained from PAsp(DET) with only a single charge in the side chain, suggesting facilitated multivalent interactions with siRNA molecules to increase the PIC stability. The PAsp(DPT) and PAsp(TEP) PICs stable in the serum-containing media underwent an appreciably enhanced uptake into cultured cells through endocytosis, and subsequently exerted effective endosomal escape for the significant silencing of target gene expression. Notably, PAsp(TEP) PIC displayed negligible cytotoxicity in sharp contrast to the highly toxic feature of PAsp(DPT) PIC. This cytotoxicity is apparently correlated with the minimal damage to the cytoplasmic membrane of cells exposed to PAsp(TEP) at pH 7.4 evidenced from the fluorescent dye (YO-PRO-1) permeation assay. There was, in turn, a significant increase in YO-PRO-1 permeability at endosomal pH of 5.5 for PAsp(TEP)-exposed cells, indicating that PAsp(TEP) exerts membrane damage in a pH-selective manner, and eventually facilitates the translocation of siRNA-loaded PIC from the acidic endosomal compartment into the cytoplasm for effective gene silencing without any severe toxicity at physiological conditions. This acidic pH modulated enhancement in membrane damage of PAsp(TEP) may be explained by an increased protonation of the arrayed amino groups in the side chain that strongly perturb the endosomal membrane integrity. Eventually, PAsp(TEP) with a side chain array of pH-sensitive amino groups was demonstrated to be a promising component for constructing siRNA carriers exerting effective gene silencing in a less toxic context.

1. Introduction

Small interfering RNA (siRNA) enables the target-specific gene silencing through RNA interference (RNAi) machinery and has been intensely desired as a pharmaceutical agent for the treatment of various intractable diseases, such as cancers, viral infections, and genetic disorders [1]. However, siRNA has the inherent problem of poor bioavailability, such as the rapid decomposition in the body and inefficient cellular internalization. Thus, development of safe and effective delivery systems for siRNA is a current challenge for siRNA-based therapies.

Polyion complexes (PICs) formed from electrostatic interaction of polycations and anionic oligonucleotides, including siRNA, can protect oligonucleotides from enzymatic degradation and facilitate

* Corresponding author. Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. Tel.: +81 35841 7138; fax: +81 3 5841 7139.
E-mail address: kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

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their cellular uptake. These PICs present a promising candidate for oligonucleotide carriers [2–5]. The most widely studied polycations in this regard are polyethylenimine (PEI) and its derivatives, which have low pKa amino groups contributing to a facilitated endosomal escape of PICs through the so-called proton sponge effect [6,7]. Although several studies have demonstrated significant gene silencing with siRNA-loaded PICs prepared from PEI derivatives [8–10], their cytotoxic effect is frequently a concern for clinical applications [11–13]. Accordingly, polycations showing highly efficient endosomal escape and minimal cytotoxicity in clinical settings are required for the practical formulation of siRNA-loaded PICs.

In the previous study, we reported that a polyaspartamide derivative bearing a 1,2-diaminoethane unit in the side chain (PAsp(DET)) (Fig. 1) exerted efficient and low toxic translocation of its PIC with plasmid DNA (pDNA) from the endosomal compartment into the cytoplasm through pH-selective membrane disruption [14–16]. The 1,2-diaminoethane unit in the side chain of PAsp(DET) changed from a mono-protonated state to di-protonated state responding to the pH drop in the endosomal compartment, which facilitated the interaction of PAsp(DET) with the endosomal membrane. Eventually, pDNA/PAsp(DET) PIC achieved appreciable *in vivo* transfection efficiency to show a therapeutic outcome in diseased animal models [17–20]. Nevertheless, the use of PAsp(DET) in siRNA transfection resulted in no significant efficacy because of the poor stability of siRNA/PAsp(DET) PIC in serum-containing media [21,22], suggesting that the substantially decreased number of anionic charges of siRNA compared to pDNA might reduce the association force in PIC formation with PAsp(DET). Here, we report that this stability issue of siRNA-loaded PIC was overcome by constructing a repetitive array of aminoethylene units in the side chain of the polycation to exert multivalent electrostatic interactions with siRNA, retaining both endosomal escaping functions and tolerability in serum-containing medium. Furthermore, by tuning the spacer length between repeating amine units, the toxicity issue can also be managed to construct the siRNA-loaded PIC with well-balanced properties of highly specific gene silencing potential and appreciably low cytotoxicity.

2. Materials and methods

2.1. Materials

β-Benzyl L-aspartate *N*-carboxy anhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan). *N*,*N*-Dimethylformamide (DMF), dichloromethane (DCM), *n*-butylamine, diethylenetriamine (DET), tetraethylenepenatamine (TEP), dipropylenetriamine (DPT), and *N*-methyl-2-pyrrolidone (NMP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMF, DCM, NMP, *n*-butylamine, DET, TEP, and DPT were distilled before use. A luciferase-expressing mouse melanoma cell line, B16F10-Luc, was purchased from Caliper LifeScience (Hopkinton, MA). Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma–Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma Co, Ltd. (Osaka, Japan). ExGen 500 was purchased from Fermentas (Ontario, Canada). Luciferase Assay System was purchased from Promega Co. (Madison, WI). All the siRNA molecules including 5′-Cy3-labeled siRNAs were synthesized by Hokkaido System Science (Hokkaido, Japan). The sequences are as follows: firefly luciferase siRNA (sense: 5′-(Cy3-) CUU ACG CUG AGU ACU UCG AdTdT-3′, antisense: 5′-UCG AAG UAC UCA GCG UAA GdTdT-3′) and scrambled siRNA (sense: 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′, antisense: 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′).

2.2. Synthesis of poly(β-benzyl L-aspartate) (PBLA)

PBLA was synthesized by the ring-opening polymerization of BLA-NCA initiated by *n*-butylamine as previously reported [23]. Briefly, *n*-butylamine (6 μL, 0.06 mmol) in DCM (490 μL) was added to BLA-NCA (1.40 g, 4.86 mmol) dissolved in 16.4 mL of DCM/DMF (9:1 v/v). The reaction solution was stirred for 48 h at 35 °C under an argon atmosphere. The solution was precipitated in hexane/ethyl acetate (6:4 v/v) and dried under reduced pressure overnight to obtain PBLA (888 mg, yield 77%). Size exclusion chromatography (SEC) was performed to determine the molecular weight distribution (MWD) of the obtained PBLA using a TOSOH HLC-8220 equipped with TSK gel columns (SuperAW4000 and SuperAW3000 × 2, TOSHO, Japan) and an internal refractive index (RI) detector at a flow rate of 0.3 mL min^{-1} at 40 °C. NMP with 10 mL LiBr was used as an eluent. A narrow MWD (Mw/Mn = 1.02) was confirmed from the SEC (data not shown). The degree of polymerization (DP) of the PBLA was calculated to be 92 from the peak intensity ratio of the butyl protons ($CH_3CH_2CH_2CH_2$-, δ = 0.8–1.5 ppm) at the α-chain end to the benzyl protons ($C_6H_5CH_2$-, δ = 5.1 and 7.3 ppm) at the side chain in the ^{1}H NMR spectrum (concentration: 10 mg/mL, solvent: dimethyl sulfoxide-d_6, temperature: 80 °C) (data not shown).

2.3. Synthesis of a series of cationic polyaspartamide derivatives, poly{N-[N′-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), poly{N-(N′-{N″-[N‴-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}-2-aminoethyl)aspartamide} (PAsp(TEP)), and poly{N-[N′-(3-aminopropyl)-3-aminopropyl]aspartamide} (PAsp(DPT))

PAsp(DET), PAsp(TEP), and PAsp(DPT) were prepared through the aminolysis reaction of PBLA with DET, TEP, and DPT, respectively, according to the previously

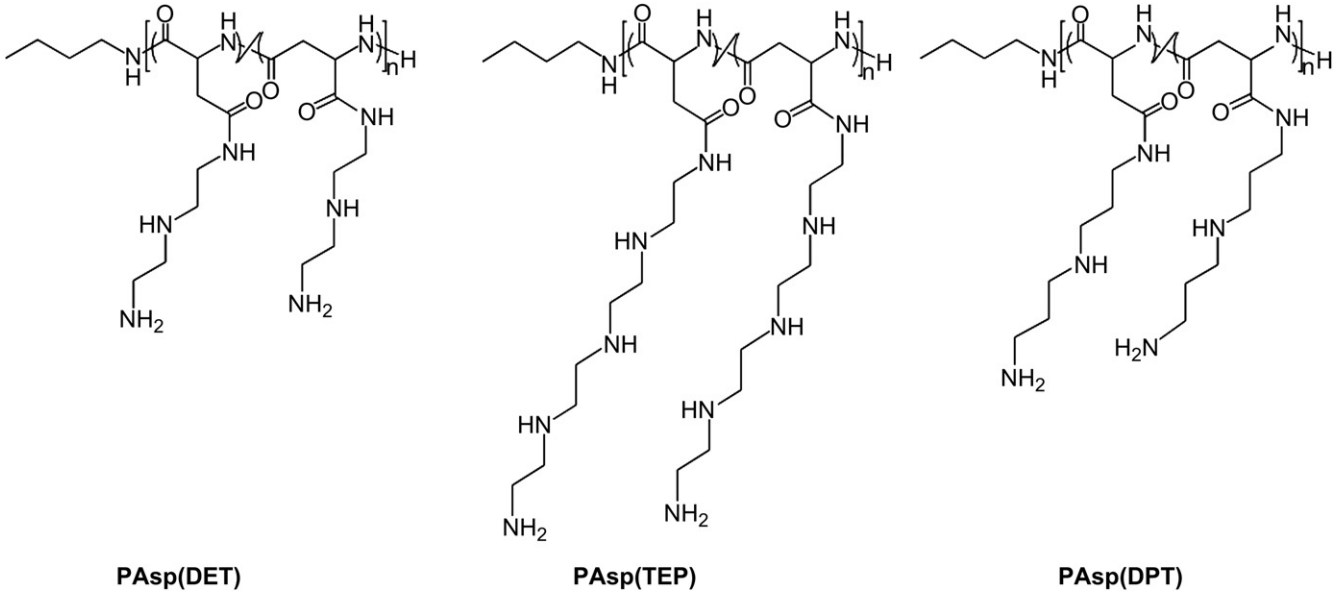

Fig. 1. Chemical structures of PAsp(DET), PAsp(TEP), and PAsp(DPT).