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An injectable cell penetrable nano-polyplex hydrogel for localized siRNA delivery

Young-Min Kim [a,b], Mi-Ran Park [a], Soo-Chang Song [a,b,*]

[a] Center for Biomaterials, Korea Institute of Science and Technology (KIST), Seoul, 136-791, Republic of Korea
[b] Department of Biomolecular Science, University of Science and Technology (UST), Daejeon, 305-350, Republic of Korea

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ABSTRACT

An approach for application of cell penetration to selective small interference RNA (siRNA) localized delivery system, cell penetrable nano-polyplex assembled hydrogel system, is presented. The cell penetrable nano-polyplex assembled hydrogelisprepared by protamine conjugation to poly(organophosphazene) and inducement of nano-polyplexes with siRNAs. After an injection of cell penetrable nano-polyplex solution into the body, it turns into a gel due to thermosensitivity of poly(organophosphazene). The gel maintains up to 4 weeks and the released 30 nm-sized nano-polyplexes from the gel induces highly effective siRNA delivery due to cell penetration. Accordingly, the new system shows a high gene silencing efficiency on only the target site in long-term with a single injection.

1. Introduction

Small interference RNA (siRNA) has been greatly expected as an alternative therapeutics of treatments for its ability to selectively down-regulate specific disease-related proteins. Despite its potential for disease treatments, siRNA is not readily applicable in clinical trials due to several obstacles such as *in vivo* instability of siRNAs, difficult cell attachment owing to ionic repulsion by the cell surface, difficult intra-cellular delivery owing to its hydrophilic property and the existence of endolysosmes which are characterized by low pH and other limiting factors within the cells [1,2]. Table 1

Cell penetrating peptides (CPPs) are short and basic amino acids rich peptides, which possess the ability to penetrate cell membrane. Because of their penetrating ability, they have been utilized for intra-cellular delivery of siRNA. Nam et al. found that more than 70% of inhibited expression of Fas protein compared to the control group in H9C2 cells after treatment of Fas siRNAs with TAT peptide, which is a type of CPPs [3,4]

In spite of successful delivery of siRNAs using CPPs into the cell *in vitro*, the applications of CPPs for siRNA delivery *in vivo*, especially in systemic delivery, are limited because CPPs are not selective in penetrating neoplastic and non-neoplastic cells. Even though selective delivery is required for the application of CPPs in clinical trials to reduce effects to normal cells, the application of targeting moiety and CPPs into one carrier would be contradictory because the carriers enter the cell *via* one single pathway, not both penetration and endocytosis [5,6]

To enable selective delivery of siRNAs by cell penetration, we used polyplex hydrogel, which we have recently designed as an injectable local delivery system [7]. In brief, a complex of anionic-charged siRNA and the polyethylenimine (PEI) conjugated polymer in polyplex solution was turned into polyplex hydrogel after injection into body by increased temperature. The polyplex hydrogel released nano-sized polyplexes that showed gene silencing efficiency for one month, but the effect was low.

We supposed that the conjugation of CPPs to nano-polyplex hydrogel may have the following merit; that of enhancing the gene silencing efficiency of released nano-polyplexes by way of injectable polyplex hydrogel system *via* CPPs and selective therapy by localization. Moreover, the time-dependent release of polyplexes from hydrogel by degradation may have helped the long-term delivery of siRNAs. (Scheme 1)

Herein, we designed and synthesized a cell penetrable nano-polyplex assembled injectable poly(organophosphazene) hydrogel to induce sustained intra-cellular delivery of siRNAs with high gene silencing efficiency *via* cell penetration. Protamine, a CPPs, and a membrane-translocating material which had been approved by Food and Drug Administration (FDA) was conjugated to carboxyl group of functionalized poly(organophosphazene) by an amide linkage. We examined the complexation, and their physicochemical

* Corresponding author. Center for Biomaterials, Korea Institute of Science and Technology (KIST), Seoul, 136-791, Republic of Korea. Tel.: +82 2 958 5123; fax: +82 2 958 5308.
E-mail address: scsong@kist.re.kr (S.-C. Song).

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Table 1
The compositions of synthesized poly(organophosphazenes).

Compound	Structure[a]	T_{ass}[b] [°C]	T_{max}[c] [°C]	$V_{37\cdot c}$[d] [Pa s]	V_{max}[e] [Pa s]	Mw ($\times 10^4$)
Polymer **1**	$[NP(IleOEt)_{1.33}(AEtOH)_{0.23}(AMPEG550)_{0.44}]_n$	5	6.8	2.5	10	3.41
Polymer **1-1**	$[NP(IleOEt)_{1.27}(Acid)_{0.29}(AMPEG550)_{0.44}]_n$	11.8	20.8	12.5	1360	5.31
Conjugate **1**	$[NP(IleOEt)_{1.27}(Acid)_{0.27}(pro)_{0.02}(AMPEG550)_{0.44}]_n$	11.8	25.9	327.5	982.5	1.8

[a] The substituted ratios were determined by ^{1}H-NMR. Viscosity was measured at 10 wt% of polymer concentration in PBS (pH 7.4)
[b] The association temperature at which the viscosity starts to increase sharply
[c] The temperature at which viscosity reaches the maximum value
[d] Viscosities at 37 °C and T_{max}
[e] Viscosities at 37 °C and T_{max}

properties and gene silencing efficiency and mechanism *in vitro*. We further investigated siRNA retention and anti-tumor effects of released cell penetrable nano-sized polyplex from polyplex hydrogel *in vivo* to determine long term anti-tumor effect mediated by siRNA delivery.

2. Experimental section

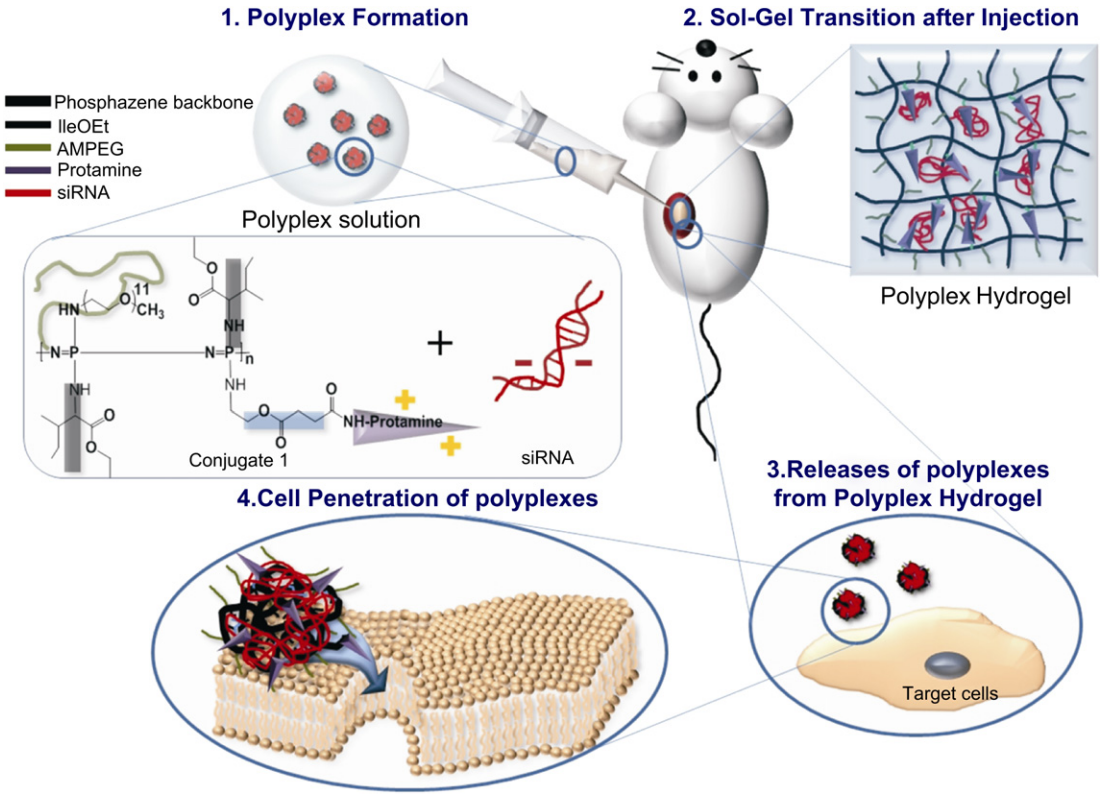
2.1. Materials

Hexachlorocyclotriphosphazene was acquired from Sigma–Aldrich (St. Louis, MO, USA)and purified by sublimation at 55 °C under vacuum (about 0.1 mmHg).Poly(dichlorophosphazene) was prepared as described previously. α-Amino-ω-methoxy-poly(ethylene glycol) with molecular weight of 550 (AMPEG 550) was prepared by a published method [16]. L-Isoleucine ethyl ester hydrocholoride (IleOEt•HCl) was purchased from A&Z food additives (HangZhou, China). Tetrahydrofuran (THF) was dried by reflux over sodium metal and distilled, and triethylamine (TEA) was distilled over barium oxide under dry nitrogen. 2-aminoethanol (AEtOH, purified by redistillation, ≥ 99.5%) was obtained from Sigma–Aldrich. Protamine sulfate (PS) was purchased from Wako (Osaka, Japan). All other reagents were purchased from commercial suppliers and used as received. All animal experiments were approved by Animal Care Ethnic Committee (ACEC) of Korea Institute of Science and Technology (KIST).siRNAs of green fluorescent protein (GFP) and vascular endothelial growth factor (VEGF) were obtained from Samchully Pharmaceutical Company (Daejeon, Korea).

GFP sense = 5′-GUUCAGCGUGUCCGGCGAGTT -3′
GFP anti-sense = 5′-CUCGCCGGACACGCUGAACTT -3′
VEGF sense = 5′-GGAGUACCCUGAUGAGAUCTT -3′
VEGF anti-sense = 5′-GAUCUCAUCAGGGUACUCCTT -3′

2.2. Synthesis of aminoethanol functionalized poly(organophosphazenes) (polymer **1**)

All reactions were carried out under an atmosphere of dry nitrogen using the standard Schlenk-line techniques. Aminoethanol functionalized poly(organophosphazene) was synthesized according to the procedure of Scheme 2, which was a modified version of the protocol described in a previous report [17]. To describe in detail, IleOEt·HCl (16.89 g, 86.29 mmol) suspended in anhydrous THF (200 ml) containing TEA (42.09 ml, 302.01 mmol) was added slowly to poly(dichlorophosphazene) (8.00 g, 69.03 mmol) dissolved in dry THF (200 ml). The reaction mixture was stirred at dry ice bath for 12 h and then at room temperature for 36 h. To this mixture, AEtOH (1.25 g, 20.71 mmol) dissolved in dry THF (50 ml) containing TEA (5.77 ml, 41.42 mmol) and AMPEG 550 (25.63 g, 46.60 mmol)dissolved in dry THF (150 ml) containing TEA (22.73 ml, 163.09 mmol)were added. The reaction mixture was stirred at room temperature for 24 h and then at 40–50 °C for 24 h. The reaction mixture was filtered; the filtrate was concentrated and poured into *n*-hexane to obtain a precipitate, which was re-precipitated twice in the same solvent system. The polymer product was further purified by dialysis with a dialysis membrane (Spectra/Por®, MWCO: 10–12 kDa) against methanol at room temperature for 4 days and distilled water at 4 °C for 4 days. The dialyzed solution was freeze-dried to obtain polymer 1. Yield: 74%. ^{31}P NMR ($CDCl_3$), δ (ppm):



Scheme 1. A schematic diagram of the cell penetrable nano-polyplex assembled hydrogel for effective localized siRNA delivery.