Injectable Polyplex Hydrogel for Localized and Long-Term Delivery of siRNA

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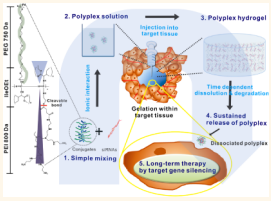
ABSTRACT Here, we describe a concept for localized and long-term delivery of short interfering RNA (siRNA) using an injectable polyplex hydrogel possessing thermosensitivity and biodegradability properties. We prepared a low molecular weight polyethyleneimine—poly(organophosphazene) conjugate as a thermosensitive and cationic polymer that has a cleavable ester linkage. The conjugates formed about 100 nm sized polyplexes with siRNAs, and the polyplex solution turned into a polyplex hydrogel at body temperature *via* a hydrophobic interaction. We injected the polyplex hydrogel with siRNA of cyclin B1, an essential protein for controlling the cell cycle, into the tumor xenograft model. Polyplexes were slowly released from the polyplex hydrogel by dissolution and degradation, allowing an *in vivo* antitumor effect *via* cyclin B1 gene silencing for 4 weeks with only a single injection.



KEYWORDS: injectable polyplex hydrogel · local delivery · sustained polyplex release · long-term therapy · siRNA

Short interfering RNA (siRNA) is expected to be a next-generation drug because of its merits of having unrestricted application to any target proteins, specificity to target mRNA, a simple manufacturing process, and low cost compared to those of protein therpeutics.[1−3] However, despite this enormous therapeutic potential, effective therapy using siRNA for a sufficient period of time has been restricted due to the lack of an efficient delivery system.[4,5]

The development of a siRNA delivery system has focused on nanosized carriers *via* systemic delivery. The polyplex, which is a nanocomplex of polycations and genes, is a representative example. It can be easily prepared by simple mixing of polycations and siRNAs, protecting the loaded genes from enzymes and proteins, and facilitating intracellular cellular uptake. Among polycations, polyethyleneimine (PEI) is one of the most frequently used carriers because of its high gene delivery efficiency, although cytotoxicity has been observed for high molecular weight PEI. For this reason, in an effort to increase biocompatibility without decreasing gene delivery efficiency, several modified carriers have been suggested based on PEI such as PEI−polyethyleneglycol conjugates and degradable PEI.[6,7] However, they have a very short retention time because of their easy dispersion property, eventuating low therapeutic efficacy in target tissues. Moreover, because they are mainly administered systemically, they can also induce nonspecific transfection or systemic immune responses.[8,9]

A local delivery carrier that can intensively transmit high concentrations of siRNA to a target site for a sufficient period can overcome these drawbacks. Among the carriers, an injectable hydrogel with a stimuli-dependent sol−gel transition has several merits such as minimal invasiveness, localization possibilities, and sustained release of the loaded drug. An injectable hydrogel for gene delivery was first suggested by Krebs *et al.* in 2009.[10] They loaded siRNAs into alginate, photo-alginate, and collagen hydrogels, and the siRNA was released for 1−2 weeks *in vitro* by different types of hydrogels. Subsequently, Han *et al.* used an injectable chitosan hydrogel for localized siRNA delivery, although it released all of the siRNA within 3 days *in vitro* and required twice per week hydrogel injections to inhibit tumor growth in a xenograft model.[11] Although these studies achieved local siRNA delivery using an injectable hydrogel, the siRNA had to be physically loaded and released as a naked form, which has a very short *in vivo* half-life and low gene silencing efficiency due to no special ability to hold and deliver the siRNAs.

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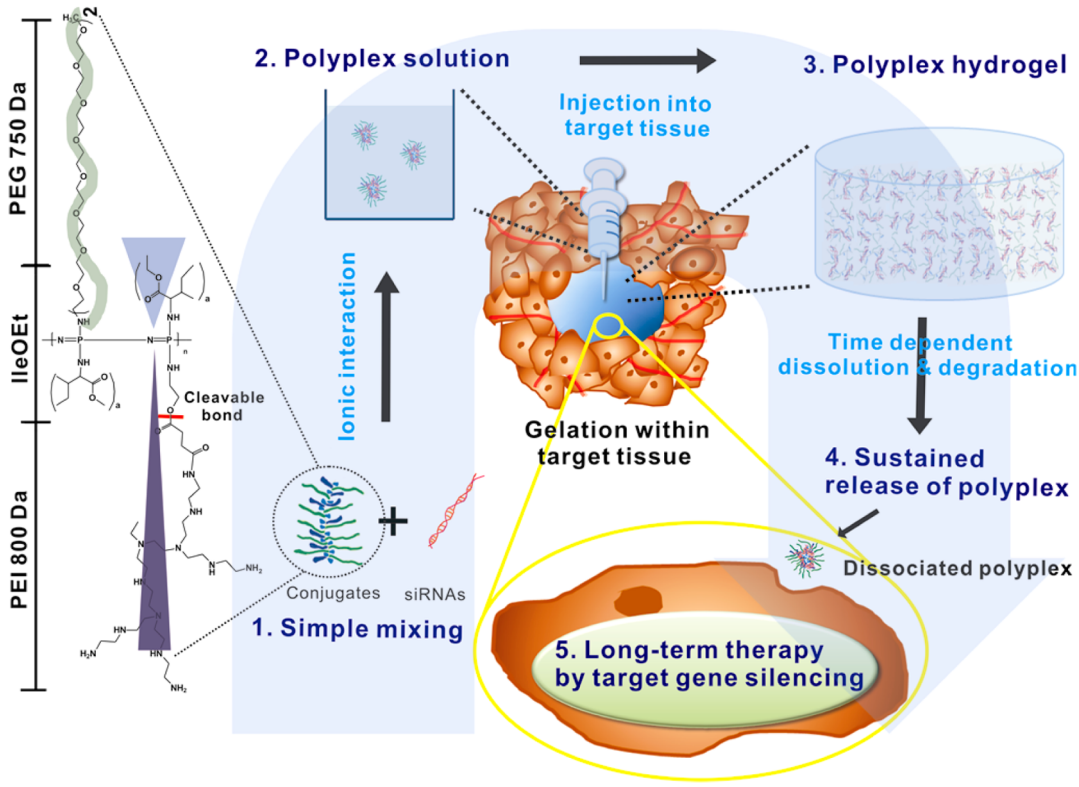
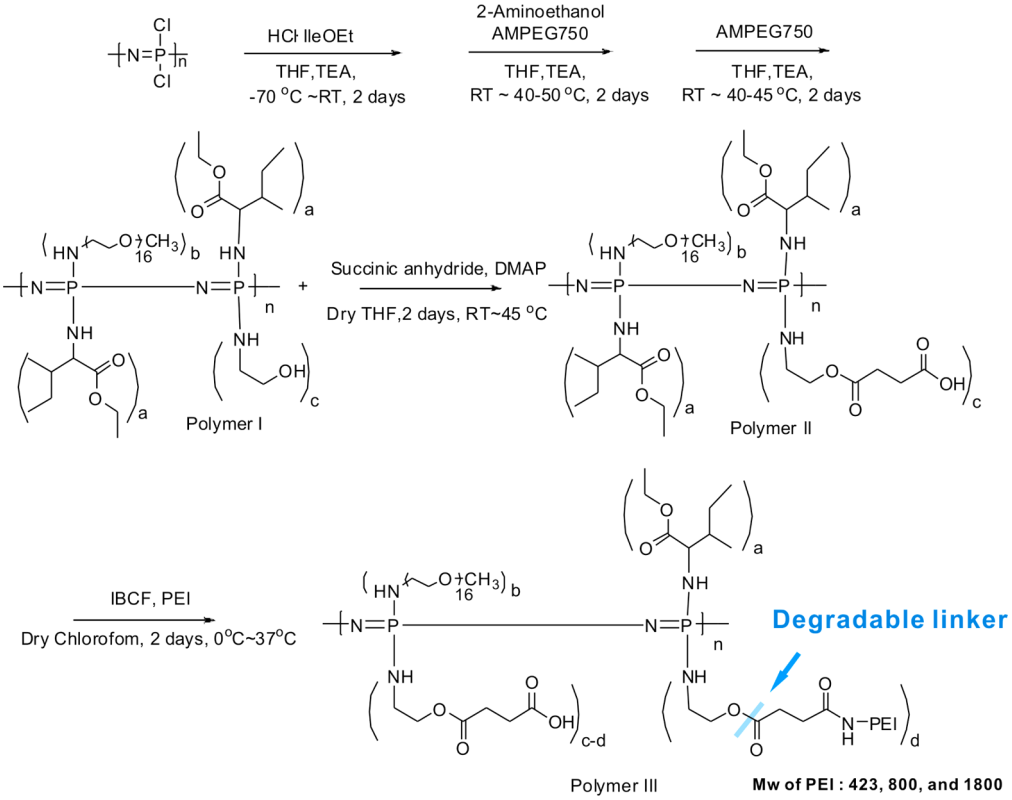


Scheme 1. Schematic concept of injectable polyplex hydrogel for localized and long-term delivery of siRNA. The polyplexes are induced by ionic interactions between L-PEI conjugates and siRNAs, and their aqueous solution transformed to a gel by injection into the target tissue. The polyplexes that have high gene silencing efficiency are released by time-dependent dissolution and degradation of the polyplex hydrogel at the target tissue, and long-term therapy by RNAi can be achieved.



Scheme 2. Synthesis scheme of L-PEI–poly(organophosphazene) conjugate.

To maintain the advantages and overcome the drawbacks of nanocomplexes and injectable hydrogels, we suggest an injectable and localizable polyplex hydrogel. Polyplex hydrogel is a complex of siRNA and a cationic polymer exhibiting temperature-dependent sol−gel transitions, and it releases nanosized polyplexes with high gene silencing efficiency for an extended period by introducing a moiety to hold and deliver the siRNAs through complexation with siRNAs. Scheme 1 shows the schematic concept of a polyplex hydrogel system. To demonstrate the concept, we synthesized low molecular weight polyethylenimine (L-PEI)−poly(organophosphazene) conjugates as cationic, degradable, and thermosensitive polymers, investigated their abilities to form a polyplex with siRNA, and characterized them with respect to gelation, degradation, polyplex release, and gene silencing efficiencies *in vitro* and *in vivo* with single injections.

RESULTS AND DISCUSSION

Synthesis of L-PEI−Poly(organophosphazene) Conjugate. The L-PEI−poly(organophosphazene) conjugate was prepared as shown in our previous report (Scheme 2).[12] First, we synthesized poly(organophosphazene), which was substituted with hydrophobic L-isoleucine ethyl ester (IleOEt) and hydrophilic α-amino-ω-methoxy-poly(ethylene glycol) (AMPEG), to enable thermosensitive gelation by hydrophobic interaction. In brief, sol−gel phase-transition behavior may be explained by the phenomenon whereby hydrophilic parts show lower critical solution temperature of the polymer. Water molecules induce hydrogen bonding in water at low temperature, while as temperature increases, the hydrogen bonding becomes broken and water molecules are released from the polymer by the increased hydrophobic interaction between molecules. Finally, hydrophobic chains associate with each other, balanced with hydrophilic swelling/dissolution, which could result in a network structure.[13] 2-Aminoethanol (AEtOH) was also substituted along with IleOEt and AMPEG to provide a hydroxyl group (polymer I). Then we esterified the hydroxyl group of AEtOH to provide a hydrolyzable ester linkage and a terminal carboxylic acid group to conjugate the PEI. Finally, L-PEI was conjugated to the newly generated carboxylic acid group of poly(organophosphazene) (polymer II) through an amide bond. We synthesized various polymers with different molecular weights of PEIs. In addition, the structures of conjugates were determined by ^{1}H-nuclear magnetic resonance spectroscopy, and the PEIs of conjugates were estimated by a ninhydrin assay (Table S1).

Preliminary Tests of the L-PEI−Poly(organophosphzene) Conjugates Composited with Different Molecular Weights of PEIs. The molecular weight of PEI is a critical factor for gene delivery, because it affects complex formation with siRNA, transfection efficiency, and cytotoxicity.

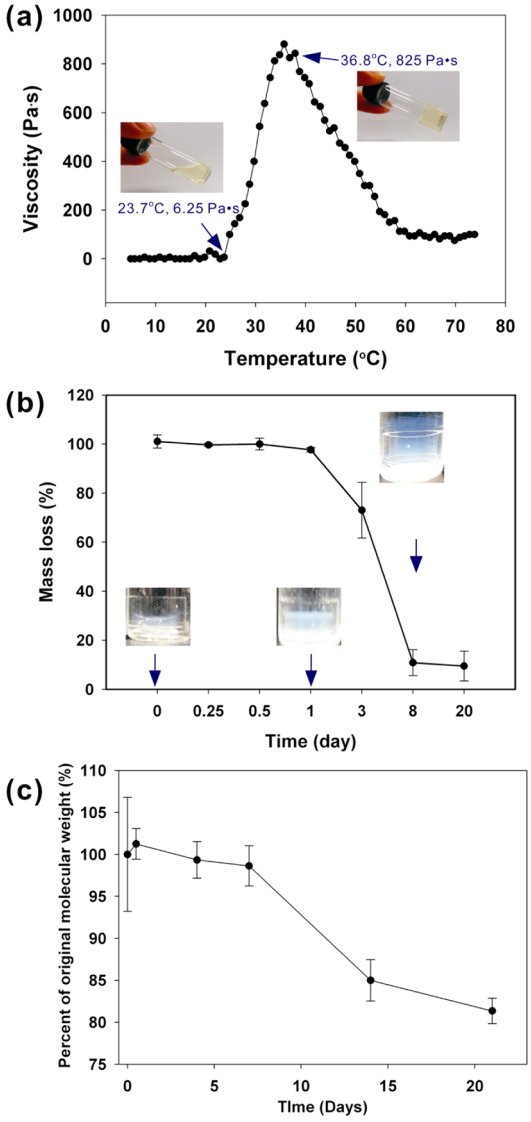


Figure 1. (a) Temperature-dependent sol−gel transition and viscosity change of 12.7 wt % aqueous conjugate 4 solution. (b) *In vitro* mass loss of conjugate 4 hydrogel with time dependency. (c) *In vitro* molecular weight decrease of conjugate 4 hydrogel with time dependency.

It is well known that complex formation ability and transfection efficiency increase by increasing the molecular weight of PEI. However, cytotoxicity also increases by increasing the molecular weight of PEI. Hence, we prepared three different L-PEI−poly(organophosphazene) conjugates with molecular weights of 423, 800, and 1800 Da, respectively, to be minimally toxic. We compared complex formation ability using siRNA, *in vitro* gene silencing efficiency, and cytotoxicity of the L-PEI conjugates as pretests (Figure. S1). Complex formation ability and gene silencing efficiency were directly proportional, whereas cytotoxicity was inversely proportional to the molecular weight of PEI, as reported previously.[14] Of the three conjugates, the PEI(800 Da) conjugate showed both high cell viability and good gene silencing efficiency;

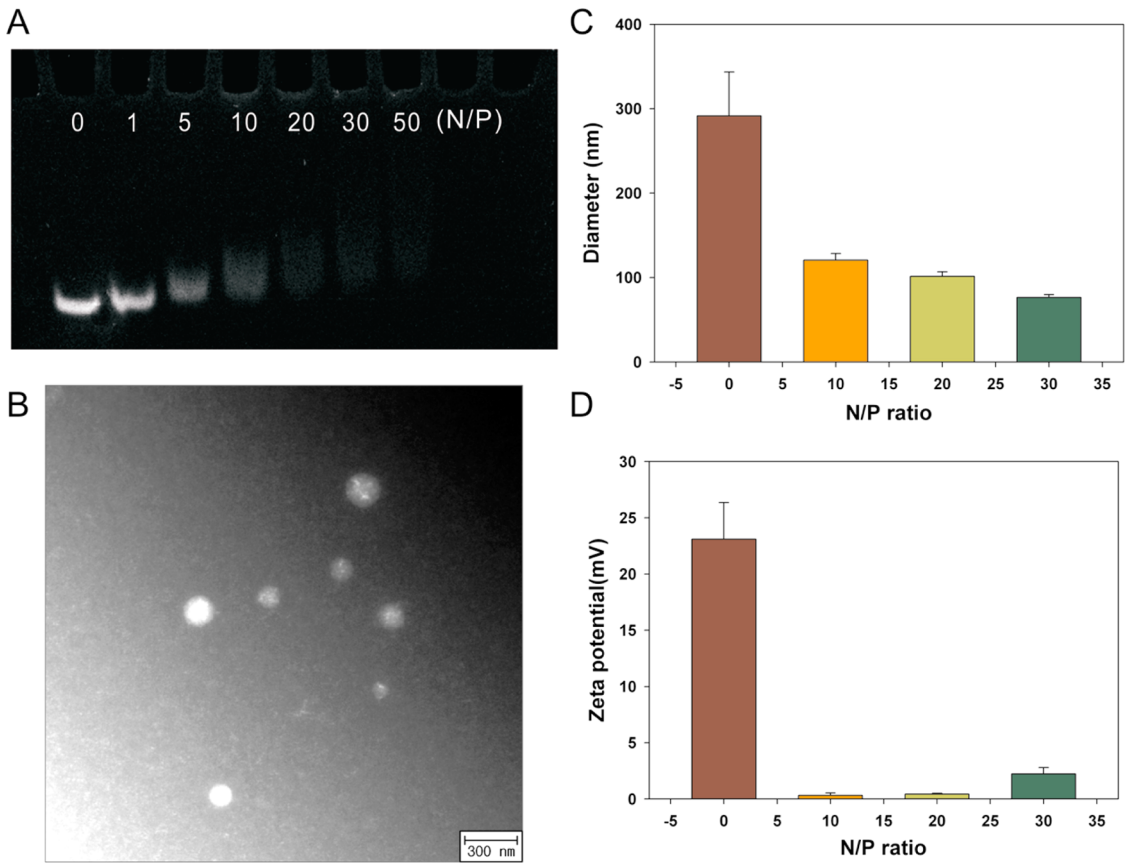


Figure 2. (a) Gel retardation assay of conjugate-4/GFP siRNA polyplexes as a function of N/P ratios. (b) TEM image of conjugate-4/VEGF siRNA polyplexes at N/P = 30. (c, d) Sizes and ζ-potentials of conjugate-4/GFP siRNA polyplexes.

hence it was selected to synthesize conjugate-**4** on large scale.

Characterization of the L-PEI−Poly(organophosphzene) Conjugate. We characterized conjugate **4**, the synthesized conjugate of L-PEI(800 Da)−poly(organophosphazene), and investigated its thermosensitivity and degradability. The structure of conjugate **4** determined by ^{1}H-nuclear magnetic resonance spectroscopy and Fourier transform infrared spectroscopy (Figure S2, Table S1) confirmed that L-PEI was successfully conjugated *via* an amide bond to the polymer at the carboxylic acid terminal. The molecular weight of conjugate **4** determined by gel permeation chromatography (GPC) was 19 106 Da.

The aqueous solution of conjugate **4** exhibited temperature-dependent sol−gel transition. The association temperature at which the viscosity of the polymer solution began to increase sharply, T_{ass}, was 23.7 °C, and the solution was transformed to a gel at 24.8 °C. Viscosity of the conjugate **4** solution was 825 Pa·s, and gelation time was within 10 s at body temperature (Table S2). Time-dependent mass loss and degradation behavior of conjugate **4** hydrogel under physiological conditions (pH 7.4, 37 °C) were observed to determine the ability for holding siRNAs. A rapid decrease of mass was observed within 8 days; however, the molecular weight of conjugate **4** hydrogel decreased slowly as time passed to 81% of the initial molecular weight at 24 day (Figure 1b,c). This indicates that the main mechanism of initial mass loss is not due to degradation but to dissolution. This is also demonstrated by the swollen hydrogel detected at day 8 in Figure 1b. These results suggest that the aqueous solution of conjugate **4** that has been synthesized can be used as an injectable and biodegradable hydrogel.

Preparation and Characterization of Polyplex between Conjugate-4 and siRNA. We confirmed the complex formation ability of conjugate-**4** with siRNA and characterized the complexes and polyplexes by measuring particle size, morphology, surface charge, stability, and gene silencing efficiency. The polycation should form an ionic complex with siRNA to protect siRNA and facilitate intracellular uptake. The formation of a polyplex using conjugate **4** and siRNA was confirmed with a gel retardation assay; a complete polyplex without free siRNA was formed from N/P = 20. The N/P value is the ratio of moles of the amine groups of cationic polymers to phosphate groups of RNA (Figure 2a).

Particles with a size less than 200 nm and a slightly positive charge are favorable for effective cellular uptake.[15,16] We used a Zetasizer with various N/P ratios to form polyplexes and measured the particle size and surface charge (Figure. 2b,c). Since conjugate **4** has special properties such as amphiphilicity, thermosensitivity, and ionicity, it is possible that there are two different driving forces, *i.e.*, hydrophobic and ionic interactions, for nanoparticle formulations between conjugate **4** and siRNAs. First, self-assembled conjugate **4**, due to the hydrophobic interaction between the hydrophobic parts of conjugate **4**, can be easily induced under aqueous conditions. Because of the presence of PEIs on the nanoparticle surfaces, the particle size of self-assembled conjugate **4** mixed with siRNAs will increase due to the attachment of siRNAs. Second, the self-complexation *via* ionic interactions between anionic succinic acid and cationic PEI800 may decrease *via* enhanced ionic interaction by siRNAs, which may result in size reduction of the particles. The particle size of conjugate **4** was 291.5 nm, and the size decreased to 76 nm after mixing with siRNAs. Thus, the nanoparticle formation by polyplexes is adequate to explain the phenomena. These phenomena were also demonstrated by the polydispersity index (PDI) values of the particles: 0.544 in the conjugate-**4**-only group and 0.340 in the polyplex group. These results indicate that mixing with siRNAs induces condensation of the particles by ionic interactions, and the condensation results in the reduction of the PDI value. Accordingly, since the ionic complexation is a major mechanism for complex formation between conjugate **4** and siRNAs, we suggest that its use, in terms of the polyplex, is reasonable for this study.

Gene Silencing Efficiency and Stability Test of Polyplexes *in Vitro*. The gene delivery efficiency of the polyplex was measured by downregulating vascular endothelial growth factor (VEGF) using VEGF siRNA for quantification of gene silencing by an Elisa kit. The conjugate-**4**/VEGF siRNA polyplex exhibited a more significantly reduced VEGF level than the groups with cells only, VEGF siRNA only, or L-PEI only, except lipofectamine. The inhibition of VEGF expression was directly proportional to the amount of VEGF siRNA used in the polyplex group. When the amount of siRNA used was increased four times, the percentage of downregulated VEGF increased from 31% to 59% (Figure 3a). Conjugate **4** showed less gene silencing effect than lipofectamine 2000 in the same amount of siRNAs; however, we assumed that a relatively high concentration of polyplexes within the region by localization can overcome the low gene silencing efficiency, and we checked the similar gene silencing effect of high quantity of polyplexes with lipofectamine 2000. Protection of the loaded siRNA from the extracellular

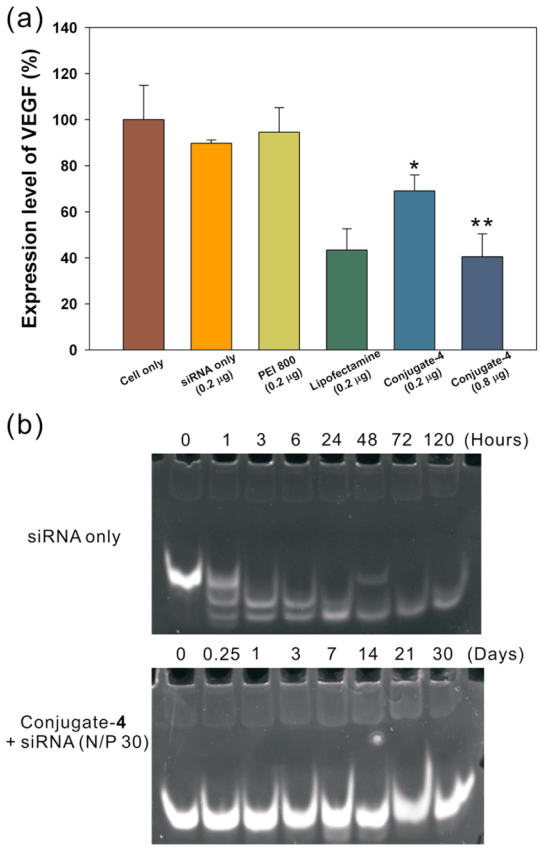


Figure 3. (a) *In vitro* VEGF gene silencing efficiency of conjugate-4/VEGF siRNA polyplex at N/P = 30 in PC-3 cells. Numbers in parentheses mean used amounts of siRNA. The asterisks indicate significant differences compared with the cell-only group (Student's *t* test). *$p < 0.08$ and **$p < 0.001$ *versus* cell only. (b) *In vitro* stability of conjugate-4/VEGF siRNA polyplex at N/P = 30 after incubation in 20% fetal bovine serum for predetermined times.

environment before its uptake into the target cell is also given by complexation. The conjugate-**4**/VEGF siRNA polyplexes showed enhanced siRNA stability in the presence of serum. In contrast to naked siRNAs, which showed degradation after 1 h, the polyplex-protected siRNA showed no severe degradation for 30 days (Figure 3b). These results indicate that the conjugate-**4**/siRNA polyplex can be used for gene therapy for their improved siRNA stability and high gene silencing efficiency.

Sustained Release of Polyplexes from Polplex Hydrogel and Their Gene Silencing Ability *in Vitro*. The aqueous solution of conjugate **4** still showed a temperature-dependent sol−gel phase transition after being mixed with the siRNA and polyplex solution. While the polyplex solution (12.7 wt %) showed a flowing sol state inner circle at 20 °C, it turned into a gel state at body temperature (Figure 4a), and the release pattern of cy5.5-tagged siRNAs from the polyplex hydrogel was estimated. The siRNA-only group showed fast elimination and loss of fluorescent intensity from siRNA; however, the

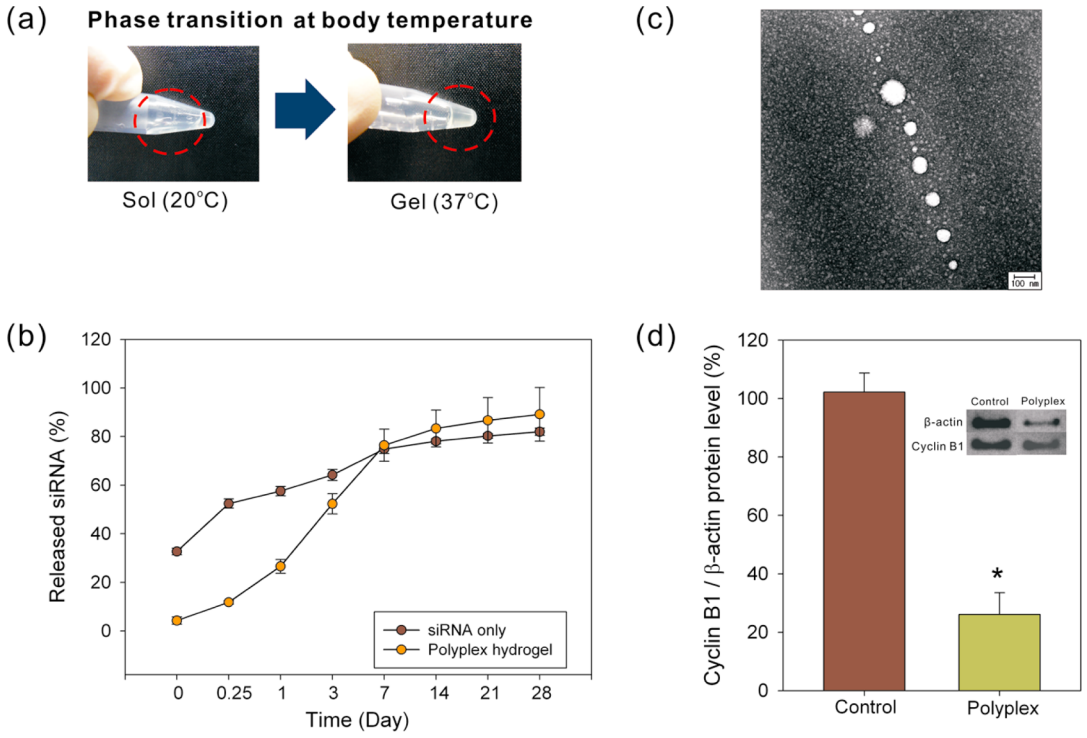


Figure 4. (a) Temperature-dependent sol−gel transition behavior of polyplex solution between siRNA and conjugate-4 at 12.7 wt %. (b) Release of dissociated nanosized polyplex from polyplex hydrogel of 12.7 wt % with cy5.5-tagged siRNA. (c) TEM image of dissociated nanosized polyplex from polyplex hydrogel of 12.7 wt % after 21 days of incubation at 37 °C. (d) Western blot analysis for checking reduced cyclin B1 protein level in the PC-3 cell line by polyplexes that was released from polyplex hydrogel of 12.7 wt % after 21 days of incubation at 37 °C. The cyclin B1 and β-actin expressions were quantified by Image J. *$p < 0.001$ *versus* control group.

polyplex hydrogel showed sustained release of siRNAs, and its pattern was consistent with the *in vitro* mass loss test of conjugate **4** hydrogel (Figure 4b). The TEM image indicated that released polyplexes from the hydrogel maintained the 50−100 nm sized diameter of spherical nanoparticles at day 21 after incubation (Figure 4c), and the released polyplexes from the hydrogel after incubation 21 days were treated with the PC-3 cell line *in vitro* to check transfection efficiency during 2 days. The reduced level of cyclin B1 compared with β-actin protein was estimated by Western blot analysis. The released polyplex showed a gene silencing effect with a more significantly reduced level of cyclin B1 than the control group: the siRNA-treated group (Figure 4d). The data suggested that conjugate **4** can be used to prepare an injectable polyplex hydrogel with siRNA and the sustainly released nanosized polyplexes from the hydrogel maintained their spherical shape and gene silencing efficiency at day 21.

Long-Term Gene Silencing Effect in *in Vivo* Xenograft Model. Finally, we transformed the polyplex solution to a hydrogel and investigated siRNA retention time and antitumor activity *in vivo*.

Prior to the siRNA-mediated antitumor activity test of the polyplex hydrogel to confirm its long-term gene silencing effect, we examined the intratumoral retention time of siRNA in the polyplex hydrogel using cy5.5-tagged siRNA in the tumor-bearing mice and compared it to that of siRNA used alone in the tumor-bearing mice. The intensity of siRNAs in the siRNA-only group, which were located within the tumor, decreased rapidly, and only very weak intensity was observed after 7 days. In contrast, the conjugate-**4**/siRNA group held fluorescence for 7 days, and a fairly high intensity was maintained for 21 days at the tumor site (Figure 5a), suggesting that the polyplex-protected siRNA from the *in vivo* environment lasted for more than 3 weeks and that long-term therapy may be possible as a result. Because we confirmed that a yellowish gel remained at the site *in vivo*, we assumed that the mass decrease of gel *in vivo* took on a different pattern from that *in vitro* because of the exposure to the water. This is consistent with the data of our previous report[17] (Figure S3a).

We next examined *in vivo* siRNA-mediated antitumor activity of the polyplex hydrogel for 30 days to confirm the effect of long-term siRNA retention. The polyplex hydrogels were prepared using cyclin B1 siRNA. Cyclin B1 is a critical mitotic regulator protein; therefore, inhibiting its expression induces cell cycle arrest at the G2 phase and apoptosis by programmed cell death.[18,19] All of the tumor-bearing mice were administered with a single intratumoral injection; however, only the group with the conjugate-**4**/cyclinB1 siRNA polyplex

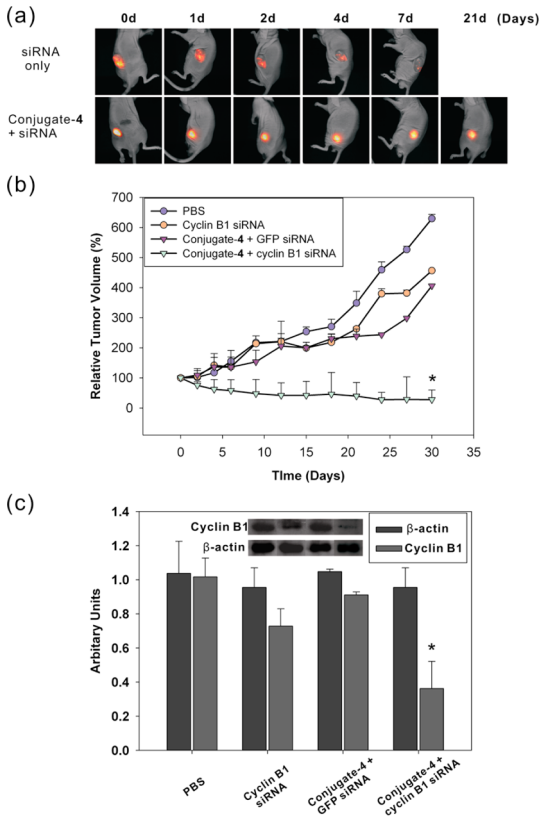


Figure 5. (a) *In vivo* siRNA retention of polyplex hydrogel of 12.7 wt % at N/P = 30 using fluorescent cy5.5-tagged cyclin B1 siRNA in PC-3 tumor xenografts. (b) Antitumor activity of conjugate-4/cyclin B1 siRNA polyplex hydrogel of 12.7 wt % at N/P = 30 in PC-3 tumor xenografts. *$p < 0.001$ *versus* the PBS-injected group. (c) Western blot analysis for intratumoral cyclin B1 levels. The cyclin B1 and β-actin expressions were quantified by Image J. *$p < 0.001$ *versus* cyclin B1 level of PBS-injected group.

showed remarkably inhibited tumor growth, and the tumor sites almost disappeared after 24 days (Figure 5b). The selective reduction of the cyclin B1 protein level in the tumor tissues after 30 days demonstrated that the antitumor activity was mediated by reduced cyclin B1 *via* RNAi (Figure 5c). To confirm tumor inhibition effects were from toxicity of the polyplex hydrogel, tissues were obtained after injection of the polyplex hydrogel at determined time intervals. Mild inflammation was present at the injected region in the initial stages, but it was not toxic enough to affect the results. Also inflammatory cells in the site disappeared with time, and few cells remained at day 21 (Figures S3b). These results indicate that the conjugate-**4**/siRNA polyplex hydrogel successfully inhibited the tumor growth by RNAi and that it is useful as a long-term formulation since, with a single injection, the sustained release of the polyplex exhibited a high gene silencing efficiency and low toxicity.

CONCLUSION

In summary, we developed, using only one material, an injectable, thermosensitive, and biodegradable polyplex hydrogel as a new concept for localized and long-term delivery of siRNA. Despite the requirement of a diagnosis before treatment with a polyplex hydrogel, many merits were suggested. Because the polyplex hydrogel was administered in a simple way, it showed extremely few signs of toxicity and achieved sustained release of the nanosized polyplex within the target region, which had a high gene silencing efficiency for an extended period. We expect that the new agent can serve as an alternative siRNA carrier for many diseases that require localized and long-term therapy, such as tumors, bone fractures, and even systemic diseases, by sustained release.

MATERIALS AND METHODS

Materials. Hexachlorocyclotriphosphazene was acquired from Sigma-Aldrich (St. Louis, MO, USA) and sublimated for purification at 55 °C under vacuum (about 0.1 mmHg). Poly(dichlorophosphazene) was prepared using sublimated hexachlorocyclotriphosphazene as described previously.[20] α-Amino-ω-methoxypoly(ethylene glycol) with a molecular weight of 750 Da (AMPEG750) was prepared by a published method.[21] L-Isoleucine ethyl ester hydrocholoride (IleOEt·HCl) was purchased from A&Z Food Additives (HangZhou, China). 2-Aminoethanol, poly(lactic-*co*-glycolic acid) (lactide:glycolide = 50:50, MW 40−70 kDa), and polyethylenimines with molecular weights of 423, 800, and 25 000 Da (PEI423, -800, and -25 000; PEI423, linear type; PEI800 and -25 000, branch type) were obtained from Sigma-Aldrich. PEI1800, branch type, was purchased from Polysciences, Inc. (Warrington, PA, USA). Lipofectamine 2000 was acquired from Invitrogen. Tetrahydrofuran (THF) and triethylamine (TEA) were dried under dry nitrogen by reflux over sodium metal/benzophenone and barium oxide, respectively. All other reagents were purchased from commercial suppliers and used as received. All animal experiments were approved by the Animal Care Ethnic Committee (ACEC), Korea Institute of Science and Technology (KIST).

siRNAs of green fluorescent protein (GFP), vascular endothelial growth factor (VEGF), and cyclin B1 were obtained from Samchully Pharmaceutical Company (Daejeon, Korea).

GFP sense = 5′-GUUCAGCGUGUCCGGCGAGTT-3′
GFP antisense = 5′-CUCGCCGGACACGCUGAACTT-3′
VEGF sense = 5′-GGAGUACCCUGAUGAGAUCTT-3′
VEGF antisense = 5′-GAUCUCAUCAGGGUACUCCTT-3′
Cyclin-B1 sense = 5′-GGCGAAGAUCAACAUGGCATT-3′
Cyclin-B1 antisense = 5′-UGCCAUGUUGAUCUUCGCCTT-3′

Synthesis of L-PEI−Poly(organophosphazene) Conjugates. All reactions were processed under an atmosphere of dry nitrogen by using standard Schlenk-line techniques. L-PEI−poly(organophosphazene) conjugates were synthesized according to the procedure of Scheme 2.

a. [NP(IleOEt)$_{1.19}$(AEtOH)$_{0.36}$(AMPEG750)$_{0.47}$]$_n$ (Polymer 1). IleOEt·HCl (9.59 g, 49.00 mmol) suspended in dry THF containing TEA was added slowly to poly(dichlorophosphazene) (4.00 g, 17.26 mmol) dissolved in dry THF. The reaction mixture was stirred in a dry ice bath for 12 h and then at room temperature for 36 h. To this mixture were added

separately AEtOH (0.63 g, 10.35 mmol) and AMPEG750 (6.99 g, 9.32 mmol), which are respectively dissolved in dry THF including TEA. The reaction mixture was stirred at room temperature for 24 h and then at 40−50 °C for 24 h. AMPEG750 (6.99 g, 9.32 mmol) dissolved in dried THF was added to the reaction mixture and 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 reprecipitated 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 for 4 days at room temperature and against distilled water for 4 days at 4 °C. The dialyzed solution was freeze-dried to obtain polymer **1**. Yield: 72%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 73H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

b. [NP(IleOEt)$_{1.14}$(Succinic acid)$_{0.38}$(AMPEG750)$_{0.48}$]$_n$ (Polymer 1-1). Succinic anhydride (0.79 g, 7.92 mmol) and 4--(dimethylamino)pyridine (DMAP) (0.97 g, 7.92 mmol), which had been separately dissolved in dried THF, were added separately into polymer **1** (9.00 g, 3.96 mmol) and dissolved in dried THF. The reaction mixture was stirred at 40 °C for 24 h. The products were dialyzed with a dialysis membrane (Spectra/Por, MWCO: 10−12 kDa) against methanol for 4 days at room temperature and against distilled water for 4 days at 4 °C. Freeze-drying was carried out after dialysis to obtain polymer **1-1**. Yield: 92%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.5−2.8 (b, 2H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 62H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

c. [NP(IleOEt)$_{1.39}$(AEtOH)$_{0.22}$(AMPEG750)$_{0.39}$]$_n$ (Polymer 2). Poly(dichlorophosphazene) (10.00 g, 86.29 mmol), IleOEt·HCl (23.81 g, 121.67 mmol), AEtOH (1.56 g, 26 mmol), and AMPEG750 (37.53 g, 50.04 mmol) were used. Yield: 72%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 73H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

d. [NP(IleOEt)$_{1.37}$(Succinic acid)$_{0.26}$(AMPEG750)$_{0.37}$]$_n$ (Polymer 2-1). Polymer **2** (27.05 g, 14.22 mmol), succinic anhydride (2.85 g, 28.44 mmol), and DMAP (3.48 g, 28.44 mmol) were used. Yield: 91%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.5−2.8 (b, 2H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 73H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

e. [NP(IleOEt)$_{1.14}$(Succinic acid)$_{0.34}$(PEI423)$_{0.04}$(AMPEG750)$_{0.48}$]$_n$ (Conjugate 1). The carboxylic acid-terminated polymer (polymer **1-1**) (1.50 g, 0.84 mmol) was dissolved in dried THF. After cooling at 0 °C, TEA (0.23 mL, 1.68 mmol) and isobutyl chloroformate (IBCF; 0.11 mL, 0.84 mmol) were added, and stirring was continued for 30 min in order to activate the carboxyl groups of the polymer. The mixture was transferred to PEI423 (1.78 g, 4.21 mmol) dissolved in distilled THF containing TEA. The reaction mixture was stirred at 0 °C for 6 h and then at room temperature for 18 h. After the reaction, the solution was concentrated and purified by precipitation with 1 M KF solution. The precipitate was dialyzed with a dialysis membrane (Spectra/Por, MWCO: 10−12 kDa) against distilled water for 3 days at 4 °C, and the dialyzed solution was freeze-dried to obtain the final product. Yield: 82%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.5−2.8 (b, 2H), 2.6−2.9 (b, 49H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 73H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

f. [NP(IleOEt)$_{1.14}$(Succinic acid)$_{0.31}$(PEI800)$_{0.07}$(AMPEG750)$_{0.48}$]$_n$ (Conjugate 2). Polymer **1-1** (1.50 g, 0.84 mmol), PEI800 (3.37 g, 4.21 mmol), IBCF (0.11 mL, 0.84 mmol), and TEA (0.23 mL, 1.68 mmol) were used. Yield: 83%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.5−2.8 (b, 2H), 2.6−2.9 (b, 74H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 62H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

g. [NP(IleOEt)$_{1.14}$(Succinic acid)$_{0.21}$(PEI1800)$_{0.17}$(AMPEG750)$_{0.48}$]$_n$ (Conjugate 3). Polymer-**1-1** (1.50 g, 0.84 mmol), PEI1800 (7.57 g, 4.21 mmol), IBCF (0.11 mL, 0.84 mmol), and TEA (0.23 mL, 1.68 mmol) were used. Yield: 86%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.6−2.9 (b, 211H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 73H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

h. [NP(IleOEt)$_{1.37}$(Succinic acid)$_{0.15}$(PEI800)$_{0.11}$(AMPEG750)$_{0.37}$]$_n$ (Conjugate 4). Polymer **2-1** (24.35 g, 10.51 mmol), PEI800 (42.04 g, 52.50 mmol), IBCF (1.36 mL, 10.51 mmol), and TEA (2.92 mL, 21.02 mmol) were used. Yield: 90%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8−1.0 (s, 6H), 1.1−1.3 (b, 3H), 1.3−1.6 (b, 2H), 1.6−1.9 (b, 1H), 2.5−2.8 (b, 2H), 2.6−2.9 (b, 74H), 2.8−3.3 (b, 2H), 3.4−3.8 (b, 73H), 3.9 (s, 1H), 4.0−4.3 (b, 3H).

Characterization of Poly(organophosphazenes). The structures of prepared polymers were estimated by measuring ^{1}H NMR (Varian Gemini-300 spectrometer operating at 300 MHz in the Fourier transform mode with $CDCl_3$) and FT-IR (Spectrum GX FT-IR, Perkin-Elmer) spectra. The amount of substituted amine groups in L-PEI was determined by ninhydrin assay (Pierce, Rockford, IL, USA). The viscosity of the aqueous conjugate **4** solutions was measured by a Brookfield RVDV-III+ viscometer between 5 and 70 °C under a fixed shear rate of 0.1 s^{-1}. The measurements were processed with a set spindle speed of 0.2 rpm and with a heating rate of 0.33 °C/min. The molecular weight (MW) of conjugate **4** was calculated by a gel permeation chromatography system (Waters 1515) with a refractive index detector (Waters 2410) and two Styragel columns (Waters Styragel HR 4E and HR 5E) connected in line at a flow rate of 1 mL/min at 35 °C. THF containing 0.1 wt % of tetrabutylammonium bromide was used as a mobile phase. Polystyrenes (MW: 1270; 3760; 12 900; 28 400; 64 200; 183 000; 658 000; 1 050 000; 2 510 000; 3 790 000) were used as standards.

***In Vitro* Mass Decrease of Polymers.** After transferring 0.3 mL of the polymer solutions (12.7 wt %) dissolved in PBS to pre-weighed Millicells (Ø: 12 mm, Millipore), the Millicells containing the polymer solution were incubated at 37 °C for 30 min in order to form a hydrogel from the solution. The hydrogel was soaked in 5 mL of buffer solution (phosphate-buffered saline (PBS) of pH 7.4) preheated to 37 °C and incubated in a water bath (KMC-1205SW1, Vision, Korea) at 37 °C under a mild shaking motion (50 rpm) for 20 days. Samples were taken at predetermined time intervals and lyophilized. The lyophilized polymers were weighed and compared with the initial weight of the polymers for determination of gel mass decrease.

***In Vitro* Molecular Weight Decrease of Polymers.** Conjugate **4**, dissolved in PBS (0.01 M, pH 7.4) at 0.4 wt %, was incubated in a water bath (KMC-1205SW1, Vision, Korea) at 37 °C under mild shaking (50 rpm). Samples were taken at predetermined time intervals and lyophilized. The molecular weights of lyophilized polymers were calculated by GPC and compared with the initial molecular weight of conjugate **4**.

Formation of Polyplexes between siRNAs and Conjugates. The polyplexes were induced by adding conjugate solution to the siRNA solution with gentle shaking and incubated at 4 °C for 30 min. All polyplexes were induced in diethylpyrocarbonate (DEPC) water and freshly prepared before use.

Gel Retardation Assay. A 0.5 μg portion of GFP siRNA was used for complexation with conjugates at predetermined N/P ratios. The induced polyplexes were loaded on 12% polyacrylamide gels, and electrophoresis was carried out at 100 mV. After 1 h, the gels were stained by ethidium bromide (EtBr), and retardation of siRNA was observed by GelDoc (MiniBIS Pro, DNR Bioimaging System, Israel).

Measurement of Size and ζ-Potential. The sizes and ζ-potentials of conjugate-**4**/GFP siRNA polyplexes were measured by a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The final concentration of siRNA was 10 μg/mL, and the samples were measured in triplicate.

Transmission Electron Microscopy (TEM). The sizes and shapes of conjugate-**4**/VEGF siRNA polyplexes at N/P 30 were observed by TEM (CM30 electron microscope, Philips, CA, USA). In addition to the freshly prepared polyplexes, we also confirmed the release of polyplex from the polyplex hydrogel; this was done by incubating the polyplex hydrogel (0.1 g) in 0.9 mL of DEPC water for 7 days at 37 °C in a water bath with slow motion (50 rpm), and the resulting supernatant was observed. One drop of sample solution was placed on a copper grid, and negative staining was done with 2 wt % uranyl acetate.

***In Vitro* Serum Stability.** For the serum stability assay, 0.2 μg of VEGF siRNA was used for complexation with conjugates at N/P = 30. The resulting polyplexes were incubated in 20% fetal bovine

serum (FBS) and 5% glucose solution for a predetermined period in a water bath at 37 °C with slow motion (50 rpm). After incubation, 2 μL of 2% sodium dodecyl sulfate (SDS) was added to each sample, and the samples were loaded on 12% polyacrylamide gel. After running the gel for 1 h, the gels were stained by EtBr and the remaining siRNA was observed by GelDoc (MiniBIS Pro, DNR Bioimaging System, Israel).

***In Vitro* Cell Viability.** NIH3T3 cells (mouse fibroblasts) were purchased from Korean Cell Line Bank (KCLB). The cells (1×10^4 cells/well) were seeded in 96-well plates (SPL, Pocheon, Korea) and incubated overnight in complete RPMI1640 culture media supplemented with 10% FBS and 1% penicillin–streptomycin. Then the culture media were replaced with serum-free media containing different concentrations of conjugates and PEI25K. After 24 h, the media were changed to plain media containing thiazolyl blue tetrazolium bromide (MTT, 98%, Alfa Aesar, Ward Hill, MA, USA; 100 μg/well) and incubated for 3 h in order to form formazan crystals. After the removal of supernatants, the formazan crystals were dissolved in 100 μL of dimethylsulfoxide and the absorbance of the solution was measured at 570 nm by a Spectramax 340 microplate reader (Molecular Devices, USA).

***In Vitro* Transfection Assay by Checking the Reduction of GFP Level.** GFP-expressing 293T cells were kindly provided by Dr. Sung Man Kang (Korea University, Seoul, South Korea). The cells (1×10^4 cells/well) were seeded in 96-well black plates (SPL) and incubated overnight in complete RPMI1640 culture media. Then the culture media were replaced with serum-free media, containing different polyplexes, which was prepared using 0.04 μg of GFP siRNA. After 5 h, the media were replaced with fresh media containing FBS and then incubated for 48 h. Each well was washed with PBS, and the GFP levels were detected by an EnVision 2103 multilabel reader (Perkin-Elmer, USA).

***In Vitro* Transfection Assay by Checking the Reduction of VEGF Level.** PC-3 cells (human prostate cancer cells) were obtained from KCLB. The cells (8×10^4 cells/well) were seeded in 24-well plates (SPL) and incubated overnight in complete RPMI1640 culture media. Then the culture media were replaced with serum-free media containing different polyplexes, which was prepared using 0.8 μg of VEGF siRNA. After 5 h, the media were changed to fresh media containing FBS and incubated for 6 h. Thereafter, the media were replaced with fresh media containing FBS and incubated for a further 16 h. The VEGF level of each group was quantified by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) using cell culture supernatants. The percentage of VEGF levels was normalized with the amounts of total protein, which was quantified by the bicinchoninic acid (BCA) assay.

***In Vitro* siRNA Release from Polyplex Hydrogel.** A 0.13 μL amount of the polyplex gel (50 μg of siRNA in conjugate **4** solution (final concentration: 12.7 wt %) was placed in a 1.5 mL polystyrene tube. The solution in the tubes changed to a gel state after incubation at 37 °C for 30 min. A 1 mL amount of DEPC water at 37 °C was added to the tube samples, and the complex gel released polyplexes. At a predetermined time, 500 μL of sample was obtained and 500 μL of fresh DEPC water was added. The amount of relased siRNA was estimated by the correlation between quantification using the RiboGreen assay and the fluorescent intensity of cy5.5-tagged siRNA showing dose-dependence. Decreased intensity through physical shielding by the complex with polymer was deduced from the fluorescent intensity, and the fluorescent intensity was converted to quantification of siRNA by correlation.

***In Vitro* Gene Silencing Efficiency Test of Released Polyplexes.** PC-3 cells (5×10^4 cells/well) were seeded in 24-well plates (SPL) and incubated overnight first in complete RPMI1640 culture media. Then the culture media were replaced with serum-free media, containing different released polyplexes including 0.8 μg of cyB1 siRNA. After 5 h, the media were changed to fresh media containing FBS and incubated for 48 h. The percentage of cyclin B1 levels was normalized with the amounts of total protein, which was quantified by BCA assay. For Western blot, 8 μg of protein was separated on SDS-PAGE, transferred onto a nitrocellulose membrane, blocked, and then incubated overnight with each of cyclin B1 mouse monoclonal antibody and β-actin mouse monoclonal antibody (Santa Cruz Biotechnology, CA, USA). After washing, the membrane was incubated with horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology), and the bands were visualized using the ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). The cyclin B1 expression was normalized with β-actin, and the band intensities were quantified by Image J software.

***In Vivo* siRNA Retention.** The 4.5×10^6 cells of PC-3 in 100 μL of PBS were injected into the dorsal subcutis of Balb/c nude mice (6 weeks, male, from Orient Bio, Korea). When the mean volume of tumors reached approximately 100 mm^3 (length $\times$ width $\times$ height $\times$ π/6), the solutions of conjugate-**4**/cy5.5-tagged cyclin B1 siRNA polyplex (13 μL) and siRNA solution (13 μL) were injected intratumorally. A 50 μg amount of siRNA was used in each group. The intensity of cy5.5 was checked at predetermined time intervals by the Kodak Image Station 4000MM digital imaging system (Carestream Health, New Haven, CT, USA).

***In Vivo* Antitumor Activity.** The 4.5×10^6 cells of PC-3 in 100 μL of PBS were injected into the dorsal subcutis of Balb/c nude mice (8 weeks, male, from Orient Bio, Korea). When the volume of tumor reached around 50 mm^3 (length $\times$ width $\times$ height $\times$ π/6), the mice were divided into four groups (1. PBS only, 13 μL; 2. cyclin B1 siRNA only, 13 μL; 3. conjugate-**4** + GFP siRNA, 13 μL; 4. conjugate-**4** + cyclin B1 siRNA, 13 μL). Then 50 μg of siRNA was injected into each experimental group (n = 5) intratumorally, and body weights and tumor volumes were monitored for a month at predetermined time intervals. The tumor size was measured using a caliper, and tumor volume was calculated by length $\times$ width $\times$ height $\times$ π/6. At day 30, the mice were sacrificed and tumor tissues dissected. The tumor tissues were homogenized and centrifuged to obtain supernatant for further analysis. Total proteins were quantified by BCA assay. For Western blot, 20 μg of protein was separated on SDS-PAGE, transferred onto a nitrocellulose membrane, blocked, and then incubated overnight with each of cyclin B1 mouse monoclonal antibody and β-actin mouse monoclonal antibody (Santa Cruz Biotechnology, CA, USA). After washing, the membrane was incubated with horseradish peroxidase-labeled goat anti-mouse IgG secondary andibody (Santa Cruz Biotechnology), and the bands were visualized using the ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). The cyclin B1 expression was normalized with β-actin, and the band intensities were quantified by Image J software.

***In Vivo* Toxicity Test.** The conjugate **4** solution (12.7 wt %, 100 μL) was injected into the dorsal subcutis of balb/c mice (6 weeks, female, from Orient Bio, Korea). These mice were sacrificed, and the existence of the gel was checked at predetermined time intervals. The tissue where the conjugate **4** gel was injected was dissected and stained with hematoxylin and eosin.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Additional figures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES AND NOTES

1. Bumcrot, D.; Manoharan, M.; Koteliansky, V.; Sah, D. W. Y. RNAi Therapeutics: A Potential New Class of Pharmaceutical Drugs. *Nat. Chem. Biol.* **2006**, *2*, 711–719.
2. Dykxhoorn, D. M.; Palliser, D.; Lieberman, J. The Silent Treatment: siRNAs as Small Molecule Drugs. *Gene Ther.* **2006**, *13*, 541–552.
3. Oh, Y.-K.; Park, T. G. siRNA Delivery Systems for Cancer Treatment. *Adv. Drug Delivery Rev.* **2009**, *61*, 850–862.
4. Haupenthal, J.; Baehr, C.; Kiermayer, S.; Zeuzem, S.; Piiper, A. Inhibition of RNase A Family Enzymes Prevents Degradation and Loss of Silencing Activity of siRNAs in Serum. *Biochem. Pharmacol.* **2006**, *71*, 702–710.

5. Liu, Y.; Huang, L. Designer Lipids Advance Systemic siRNA Delivery. *Mol. Ther.* **2010**, *18*, 669–670.
6. Jiang, G.; Park, K.; Kim, J.; Kim, K. S.; Hahn, S. K. Target Specific Intracellular Delivery of siRNA/PEI−HA Complex by Receptor Mediated Endocytosis. *Mol. Pharmaceut.* **2009**, *6*, 727–737.
7. Park, M. R.; Kim, H. W.; Hwang, C. S.; Han, K. O.; Choi, Y. J.; Song, S. C.; Cho, M. H.; Cho, C. S. Highly Efficient Gene Transfer with Degradable Poly(ester amine) Based on Poly(ethylene glycol) Diacrylate and Polyethylenimine *in Vitro* and *in Vivo*. *J. Gene Med.* **2008**, *10*, 198–207.
8. Heyes, J.; Palmer, L.; Chan, K.; Giesbrecht, C.; Jeffs, L.; MacLachlan, I. Lipid Encapsulation Enables the Effective Systemic Delivery of Polyplex Plasmid DNA. *Mol. Ther.* **2007**, *15*, 713–720.
9. Sakurai, H.; Kawabata, K.; Sakurai, F.; Nakagawa, S.; Mizuguchi, H. Innate Immune Response Induced by Gene Delivery Vectors. *Int. J. Pharmaceut.* **2008**, *354*, 9–15.
10. Krebs, M. D.; Jeon, O.; Alsberg, E. Localized and Sustained Delivery of Silencing RNA from Macroscopic Biopolymer Hydrogels. *J. Am. Chem. Soc.* **2009**, *131*, 9204–9206.
11. Han, H. D.; M., E. M.; Roh., J. W.; Nishimura, M.; Lee., S. J.; Rebecca, L. S.; Menashe, B.; Gabriel, L. B.; Anil, K. S. Chitosan Hydrogel for Localized Gene Silencing. *Cancer Biol. Ther.* **2011**, *11*, 839–845.
12. Seo, B.-B.; Park, M.-R.; Chun, C.; Lee, J.-Y.; Song, S.-C. The Biological Efficiency and Bioavailability of Human Growth Hormone Delivered Using Injectable, Ionic, Thermosensitive Poly(organophosphazene)-polyethylenimine Conjugate Hydrogels. *Biomaterials* **2011**, *32*, 8271–8280.
13. Lee, B. H.; Lee, Y. M.; Sohn, Y. S.; Song, S.-C. A Thermosensitive Poly(organophosphazene) Gel. *Macromolecules* **2002**, *35*, 3876–3879.
14. Morimoto, K.; Nishikawa, M.; Kawakami, S.; Nakano, T.; Hattori, Y.; Fumoto, S.; Yamashita, F.; Hashida, M. Molecular Weight-Dependent Gene Transfection Activity of Unmodified and Galactosylated Polyethyleneimine on Hepatoma Cells and Mouse Liver. *Mol. Ther.* **2003**, *7*, 254–261.
15. Gratton, S. E. A.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. The Effect of Particle Design on Cellular Internalization Pathways. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11613–11618.
16. Joanna Rejman, V. O.; Zuhorn, Inge S; Hoekstra, Dick Size-Dependent Internalization of Particles *via* the Pathways of Clathrin- and Caveolae-Mediated Endocytosis. *Biochem. J.* **2004**, *377*, 159–169.
17. Park, M.-R.; Cho, C.-S.; Song, S.-C. *In Vitro* and *in Vivo* Degradation Behaviors of Thermosensitive Poly(organophosphazene) Hydrogels. *Polym. Degrad. Stab.* **2010**, *95*, 935–944.
18. Crombez, L.; Morris, M. C.; Dufort, S.; Aldrian-Herrada, G.; Nguyen, Q.; Mc Master, G.; Coll, J.-L.; Heitz, F.; Divita, G. Targeting Cyclin B1 through Peptide-Based Delivery of siRNA Prevents Tumour Growth. *Nucleic Acids Res.* **2009**, *37*, 4559–4569.
19. Lapenna, S.; Giordano, A. Cell Cycle Kinases as Therapeutic Targets for Cancer. *Nat. Rev. Drug Discovery* **2009**, *8*, 547–566.
20. Chun, C.; Lee, S. M.; Kim, S. Y.; Yang, H. K.; Song, S.-C. Thermosensitive Poly(organophosphazene)-paclitaxel Conjugate Gels for Antitumor Applications. *Biomaterials* **2009**, *30*, 2349–2360.
21. Bromberg, L.; Temchenko, M. Self-assembly in Aqueous Solutions of Poly(ethylene oxide)-*b*-poly(propylene oxide-)-*b*-poly(ethylene oxide)-*b*-poly(vinyl alcohol). *Langmuir* **1999**, *15*, 8633–8639.