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Gene Carrier Showing All-or-None Response to Cancer Cell Signaling

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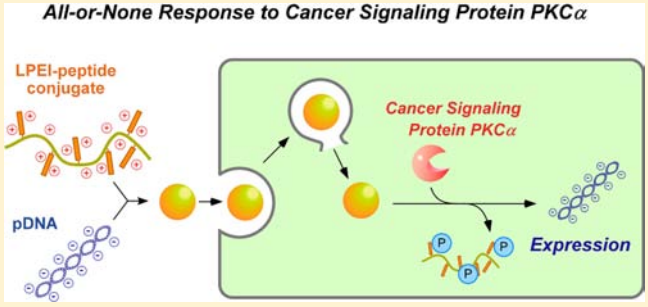
Supporting Information

ABSTRACT: In this work we designed a novel nano carrier, a linear polyethylenimine (LPEI)-peptide conjugate, for cancer-specific expression of transgenes. The conjugate was easily synthesized by using a click chemistry scheme orthogonal to the reactive side groups of the peptide, which is the substrate of protein kinase Cα (PKCα). Polyplexes of the conjugates with plasmid DNA (pDNA) were intact and stably dispersed even in the presence of cell lysate. Despite this stability, the polyplexes readily dissociated upon phosphorylation of the grafted peptides by PKCα. Because of its endosomal escape ability and adequate susceptibility to PKCα, the polyplexes showed an all-or-none type response to PKCα activity in transgene expression in vitro. The polyplexes achieved cancer tissue-specific transgene expression even for a tumor with a relatively low PKCα activity. Thus the LPEI−peptide conjugate has high potential as a nanocarrier for cancer-targeted gene therapy.



INTRODUCTION

Gene therapy is expected to be able to treat untreatable diseases such as cancers.[1−3] Synthetic gene carriers have great potential compared with viral vectors for cancer gene therapy, because they typically have low immunogenicity and are capable of facile functionalization and mass production. Therefore, various synthetic materials, including polyethylenimine (PEI),[4−7] cationized polysaccharide,[8−10] block copolymers,[11−14] and branched polymers,[15−17] have been reported as gene carriers for cancer treatment. Cancer cell-specificity and superior transfection efficiency are the most important issues for clinical applications, especially when suicide genes, which destroy the transfected cells, are employed. In this regard, active targeting through cancer cell-specific ligands has been the most widely studied strategy.[18−24] Although the active targeting improves accumulation of gene carriers to target tissues, undesirable nonspecific distribution to healthy tissues is still inevitable. Therefore, gene carriers are designed to selectively release the therapeutic genes within cancer cells by sensing the unique intracellular environments within cancer cells.

In terms of cancer cell-specific environments, we have focused on dysregulation of intracellular signaling in cancer cells. The dysregulation of signaling is frequently brought by hyperactivation of certain protein kinases (PKs) including PKC and Akt.[25−31] Among these PKs, protein kinase Cα (PKCα) can be used as a marker to distinguish cancer cells from normal cells[32] because PKCα is a key intracellular enzyme related to cancer proliferation that is hyperactivated in various cancer cells but shows very low activity in normal cells.[29−31] Recently, our group proposed a unique gene carrier that specifically responds to hyperactivation of PKCα in cancer cells to activate transgenes. This gene carrier consists of a polyacrylamide main chain and a PKCα-specific cationic peptide substrate (FKKQGSFAKKK-NH_2) as a graft chain (PPC(S), Figure 1A).[32−35] Phosphorylation of the grafted peptide, catalyzed by PKCα, reduces the cationic charges of the peptide to release the transgenes from the carriers for expression. We succeeded in PKCα-responsive reporter-gene expression both in vitro and in vivo using several cancer cell lines possessing strong PKCα activities. We previously demonstrated suppression of growth of xenografted tumors in mice using suicide gene therapy.[34] However, the above polymeric carrier PPC(S) failed to respond to some cancer cell lines with relatively low PKCα activity because of the low transfection efficacy of the polyplex. The high sensitivity of the polyplex to PKCα is crucial to practical applications because actual cancer tissues in patients are composed of many types of cells, possessing a wide range of PKCα activity.[36,37]

Here, we designed a new PKCα-responsive gene carrier, a linear PEI (LPEI)−peptide conjugate (LPEI(S), Figures 1A). LPEI is one of the most widely employed cationic polymeric gene carriers because of its high transfection ability via endosomal escape[4−6] and minimal toxicity after systemic injection.[7] We established the straightforward synthesis of the LPEI−peptide conjugate through click chemistry. The resulting

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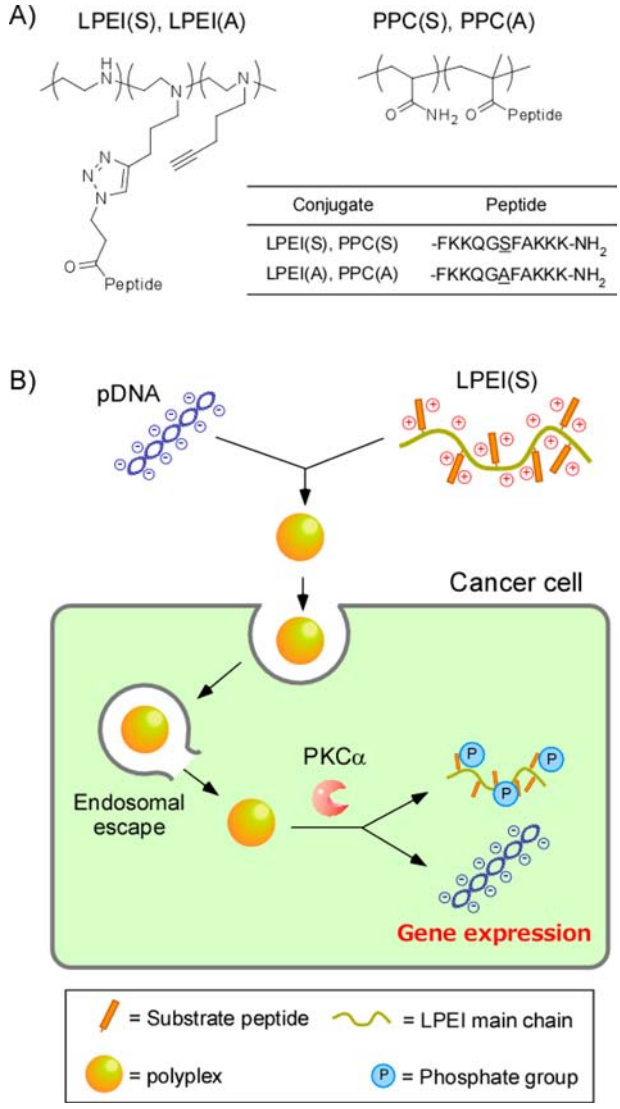

Figure 1. (A) Chemical structure of the conjugates used in this study. (B) Illustration of the cancer cell-targeted gene delivery system responding to intracellular PKCα which is hyperactivated in various cancer cells and tissues. The LPEI–peptide conjugate (LPEI(S)) comprises a LPEI main chain and a PKCα-specific peptide substrate side chain. Transcription of the pDNA is suppressed by the formation of a polyplex. After the phosphorylation reaction with PKCα, however, the pDNAs are released from the polyplex because of the decrease in net cationic charge, leading to transgene expression.

conjugate exhibited a sensitive response to intracellular PKCα activity during transgene expression, achieving 100-times higher PKCα response than our previous PPC carrier in vitro. The conjugates successfully showed a cancer tissue-specific expression even for cancer tissue with relatively low PKCα activity.

RESULTS AND DISCUSSION

Synthesis of LPEI–Peptide Conjugates. Modification of peptides on polymers is often troublesome because reactive side groups of the peptides disturb the selective reaction. To address the issue, we employed click chemistry, copper(I)-catalyzed azide–alkyne cycloaddition reaction, which is known to be an orthogonal reaction to reactive groups of biomolecules.[38,39] First, 20 mol % of the secondary amino groups of LPEI (MW = 25 000) were modified with 5-chloro-1-pentyne to obtain LPEI–Pentyne (Scheme 1). The peptide substrate modified with the azide group on the *N*-terminus was then conjugated with LPEI–Pentyne by the cycloaddition reaction (Scheme 1). We also synthesized a negative control polymer, LPEI(A), in which the phosphorylation site, a serine residue, was replaced with an alanine residue (Figure 1A). The peptide contents of LPEI(S) and LPEI(A) were determined by a trinitrobenzenesulfonic acid (TNBS) assay to quantify the primary amino groups on the grafted peptides.[40] Both conjugates showed relatively high peptide contents (>6 mol % monomeric unit) as represented in Table 1. For comparison, previous PKCα-responsive polymers, PPC(S) and PPC(A), were also synthesized as described previously.[32]

pDNA Condensation Ability. We compared the pDNA condensation ability of each polymeric carrier using an ethidium bromide (EtBr) exclusion assay.[41] LPEI(S) and LPEI(A) showed the same tendency for pDNA condensation at each N/P, where N is the total amine number in each carrier and P is the total phosphate number in pDNA (Figure S2, Supporting Information). Figure 2A shows the changes in relative fluorescence intensity (RFI) of EtBr bound to polyplexes at varying N/P ratios. LPEI(S) required a much higher N/P ratio than PPC(S) to reach a stable RFI for complete condensation of pDNA (Figure 2A). Interestingly, when the N/P ratio was converted to the N_{pep}/P ratio, where N_{pep} is the number of amine groups in the peptide, the RFI profile of LPEI(S) and PPC(S) became superimposable (Figure 2B). This result clearly shows that LPEI(S) binds to pDNA selectively through the grafted peptides and cationic charges of the LPEI backbone do not contribute to the condensation. This property of LPEI(S) is critical for our transgene regulation system, where the binding of carriers to pDNA should be governed by the cationic charges of the peptide. As shown in Figure 2C, the binding ability of LPEI–Pentyne with pDNA was much lower than that of the original LPEI. This is probably because of shielding of the cationic charges on the LPEI backbone by the bulky pentyne groups (Figure 2C). The weak binding ability of LPEI–Pentyne was also revealed by a gel electrophoresis mobility assay (Figure S3, Supporting Information).

Phosphorylation of Conjugate with PKCα for Polyplex Dissociation. We examined the phosphorylation of the grafted peptides of LPEI(S) by PKCα using a coupled-enzyme assay that can monitor the consumption of ATP during the phosphorylation reaction.[42] As shown in Figure 3A, a decrease in absorption at 340 nm (A_{340}) resulting from ATP consumption was observed in LPEI(S) but not in LPEI(A). The time course for A_{340} decrease in LPEI(S) was similar to that in the original substrate peptide, showing sufficient reactivity of the grafted peptides.

We then confirmed whether the phosphorylation of the grafted peptide with PKCα could dissociate a polyplex prepared at an N/P ratio of 10. The dissociation of the polyplex was monitored by changes in light scattering intensity (LSI) upon addition of PKCα (Figure 3B). The LSI of LPEI(A) polyplex was nearly constant, while that of LPEI(S) polyplex steeply decreased with time, suggesting that LPEI(S) polyplex was dissociated by the phosphorylation reaction with PKCα.

Stability of Polyplexes in Cell Lysate. We investigated the stability of the polyplex of LPEI–peptide conjugates and PPC in diluted cell lysate containing various macromolecules that could possibly lead to dissociation of the polyplex by

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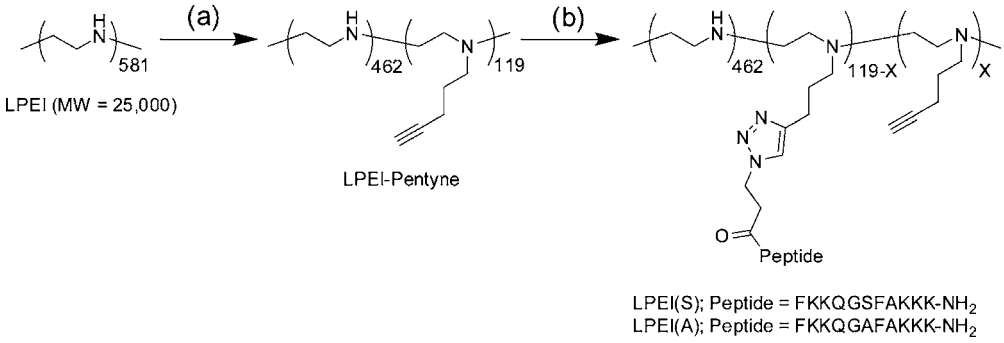
Scheme 1. Synthesis Scheme for LPEI(S) and LPEI(A)[a]



[a]Reagents and conditions: (a) 5-chloro-1-pentyne, DBU, and dry DMSO at 50°C; (b) N-terminus azido-peptide, copper(II) pentahydrate, sodium ascorbate, and H_2O/ethanol = 1/1 at RT.

Table 1. Molecular Parameters of Polymers

samples	peptide content/mol % monomeric unit	peptide no./ chain	M_w/ 10^4 g/mol
LPEI(S)	6.1[a]	35	8.2[c]
LPEI(A)	7.2[a]	42	9.0[c]
PPC(S)	2.9[b]	61	23[d]
PPC(A)	2.8[b]	55	20[d]

[a]Determined by TNBS assay. [b]Determined by elemental analysis. [c]Calculated from peptide and pentyne contents and M_w of parent LPEI. [d]Determined by GPC as described previously.[32]

exchanging with pDNA.[43] Each polyplex was first prepared in 10 mM HEPES buffer. The diameter and ζ-potential of each polyplex are summarized in Table S1, Supporting Information. PPC(S) polyplex prepared at an N/P ratio of 4 had a diameter of 122 ± 2 nm. When this polyplex was mixed with diluted cell lysate, the polyplex size gradually increased to exceed 1 μm and the LSI of the polyplex decreased simultaneously (Figure 4). These results correspond to loosening or dissociation of the PPC(S) polyplex. In contrast, the LPEI(S) polyplex prepared at an N/P ratio of 10 (N_{pep}/P = 2.3) maintained both its original diameter (~160 nm) and LSI after mixing with cell lysates. The excellent tolerance of the LPEI(S) polyplex to macromolecules contained in the cell lysate was probably due to the stronger binding between LPEI(S) and pDNA because of the high density of the grafted cationic peptides. These characteristics are critical for suppressing undesirable transgene expression in normal cells caused by nonspecific dissociation of the polyplex through the exchange reactions with intracellular macromolecules. It is worth emphasizing that despite this stability, the LPEI(S) polyplex was readily dissociated by phosphorylation with PKCα as demonstrated in Figure 3.

All-or-None Type Response of Transgene Expression to Abnormal PKCα Activity in Vitro. Transgene activation in response to intracellular PKCα was evaluated for polyplexes of LPEI–peptide conjugates and PPCs by using luciferase encoded pDNA. Polyplexes prepared at various N/P ratios were transfected into HepG2 human hepatoma cells, where PKCα is activated.[32,35] PPC polyplexes showed the best performance at an N/P ratio of 4, where PPC(S) expressed 4 times more luciferase than PPC(A), which was not responsive to PKCα (parts A and B of Figure 5). Surprisingly, LPEI(S) exhibited 145 times more transgene expression than LPEI(A) at an N/P ratio of 10 (parts C and D of Figure 5). Figure 6A summarizes the results of in vitro transfection by using LPEI–peptide conjugates and PPCs at their optimal N/P ratios.

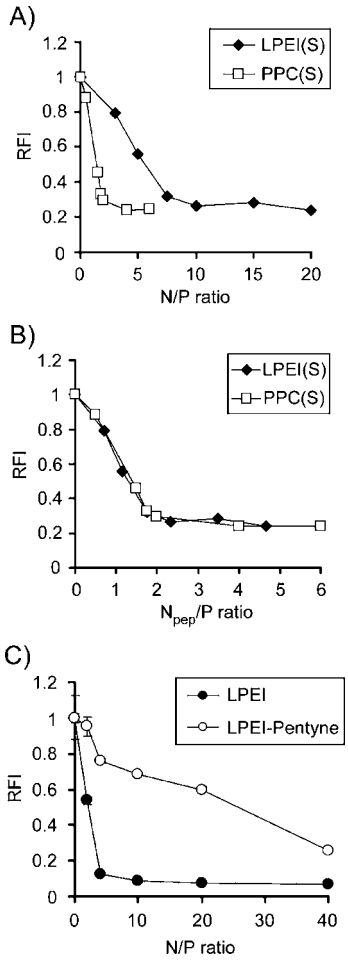


Figure 2. (A) Polyplex formation between LPEI(S) or PPC(S) and pDNA. The x-axis is the N/P [(total amine in polymer)/(total phosphate in pDNA)] ratio. RFI, relative fluorescence intensity. (B) Binding of LPEI(S) or PPC(S) with pDNA when the N/P ratio is converted to an N_{pep}/P [(amine in peptide)/(phosphate in pDNA)] ratio. (C) Degree of pDNA concentration for LPEI or LPEI–pentyne.

Transgene expression of LPEI(S) was 10 times higher than PPC(S), while the undesirable expression in the negative control LPEI(A) was smaller than with PPC(A). Notably, the transgene expression of LPEI(A) was maintained at a low level for 3 days after transfection (Figure 6B), indicating the highly stable nature of this polyplex under the intracellular conditions which includes a very high concentration of macromolecules

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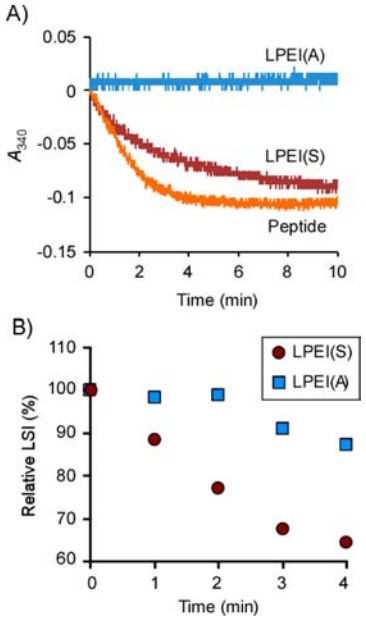

Figure 3. (A) Phosphorylation reactions of LPEI–peptide conjugates and peptide. (B) Changes of light scattering intensity (LSI) after addition of PKCα. After forming polyplexes of pDNA with LPEI(S) (solid circle) or LPEI(A) (open square) at an N/P ratio of 10, PKCα was added to polyplex dispersion and measurement was then started.

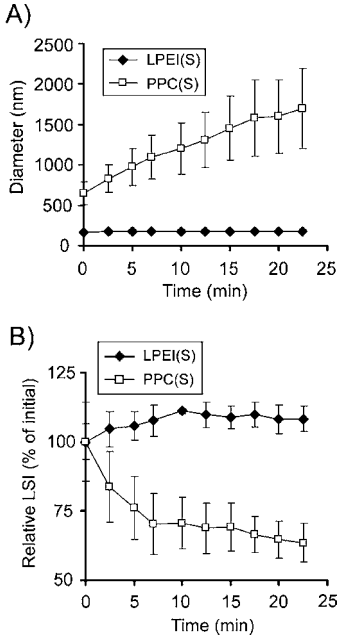

Figure 4. Change in (A) diameter and (B) LSI of LPEI(S)/pDNA and PPC(S)/pDNA polyplexes in buffers containing cellular lysates. LSI, light scattering intensity. Data are means ± SD of three independent experiments.

that are susceptible to exchange with DNA. This all-or-none type response of the LPEI–peptide conjugates is very promising for a cancer-specific gene therapy that avoids undesirable transgene expression in healthy normal cells. Although LPEI(S) polyplexes showed highly efficient transgene expression, they had no detectable cytotoxicity (Figure S4, Supporting Information). As shown in Figure 6A, the gene expression of LPEI(S) is lower than that of LPEI. This may be

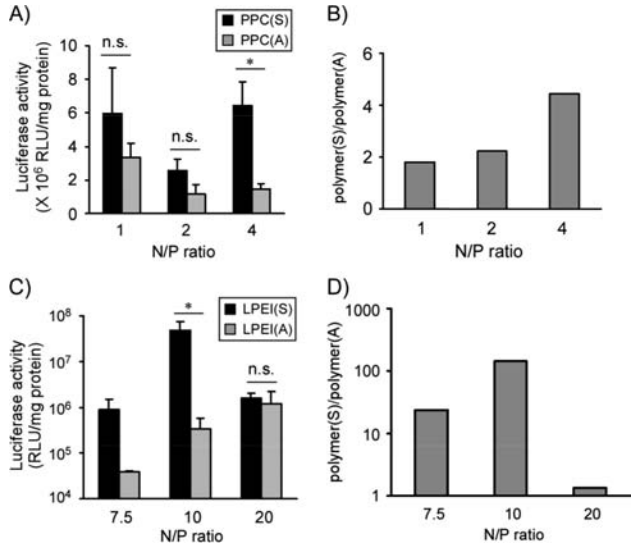

Figure 5. Transfection of PPC/pDNA and LPEI–peptide conjugate/pDNA polyplexes into HepG2 cells. (A) Optimization of N/P ratio of PPC/pDNA polyplexes. (B) Calculation of polymer(S)/polymer(A) ratio obtained from part A. (C) Optimization of N/P ratio of LPEI–peptide conjugate polyplexes. (D) Calculation of polymer(S)/polymer(A) ratio obtained from part C. Asterisk: $P < 0.05$ indicates the significant difference in luciferase activity between groups (n = 5–10). n.s.: not significant. Data are means ± SD.

explained by suppressive characteristics of LPEI(S) for gene expression comparing with LPEI due to the requirement of peptide phosphorylation for the gene expression in LPEI(S).

We examined the PKCα-responsive expression of the polyplexes in three other cancer cell lines with different PKCα activities.[32] As shown in Figure 6C, LPEI–peptide conjugates showed much higher polymer(S)/polymer(A) ratios (defined as the ratio of gene expression from polymer(S) to that from polymer(A)) than PPCs for all three cell lines. LPEI(S) exhibited the clearest response to PKCα in the U87-MG human glioblastoma cell, where the polymer(S)/polymer-(A) ratio reached more than 400, which is 100 times superior to PPCs. Because of the all-or-none type response of LPEI–peptide conjugates to PKCα, they achieved a polymer(S)/polymer(A) ratio of more than 20 even for Neuro2A (N2A), which had the lowest PKCα activity. The polymer(S)/polymer(A) ratio showed a good correlation with PKCα activity in the cell (R = 0.882).

Origin of the All-or-None Type Response of the LPEI–Peptide Conjugate. The cellular uptake of two kinds of polyplexes was evaluated by using fluorescein-labeled pDNA. The amount of cellular uptake of polyplexes with LPEI–peptide conjugates was similar to that of PPCs (Figure 7A). Therefore, much higher expression of LPEI(S) than PPC(S) did not result from the difference in the amount of the pDNA uptake. To compare the endosomal escape ability of the two kinds of polyplex types, the effect of endosomal escape inhibitor (nigericin;[44,45] NR) on the transgene expression is shown in Figure 7B. NR treatment showed almost no effect on the transgene expression of PPC(S), but significantly reduced that of LPEI(S) to the same level as the original LPEI. These results clearly show that the much higher expression of LPEI(S) is attributable to efficient endosomal escape due to the buffering capacity of the LPEI backbone in LPEI(S). LPEI(S) shows somewhat lower cellular uptake than LPEI (Figure 7A), which

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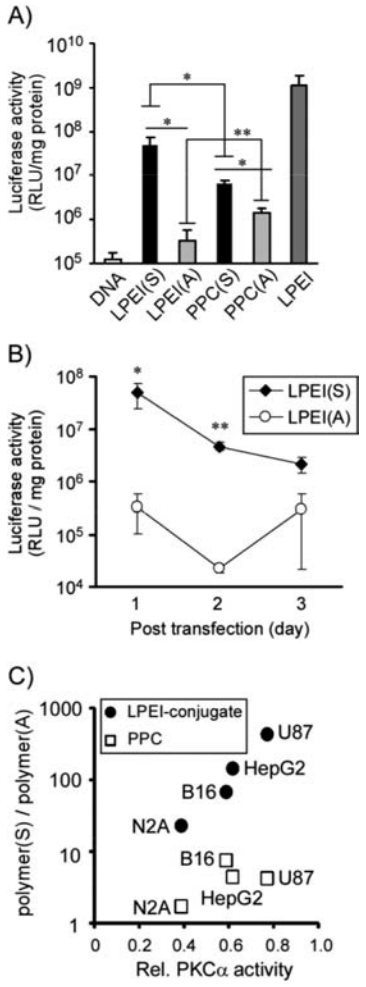

Figure 6. (A) Transfection of various polyplexes [LPEI–peptide conjugate (N/P = 10) (n = 10), PPC polymer (N/P = 4) (n = 10), and LPEI alone (N/P = 10) (n = 5)] into HepG2. Luciferase activity was detected at 24 h after transfection. (B) Time course of luciferase activity after transfection of LPEI(S)/pDNA or LPEI(A)/pDNA polyplex into HepG2 cells at an N/P ratio of 10. (C) Relationship between the relative PKCα activity of cancer cells and the transgene expression ratio of polymer(S) to polymer(A) (polymer(S)/polymer(A) ratio). Relative PKCα activity was defined as the relative phosphorylation ratio of cellular lysates measured by MALDI-TOF-MS, as described previously.[32] Asterisk, $P < 0.05$, and double asterisk, $P < 0.01$, indicate the significant difference in luciferase activity between groups. Data are means ± SD.

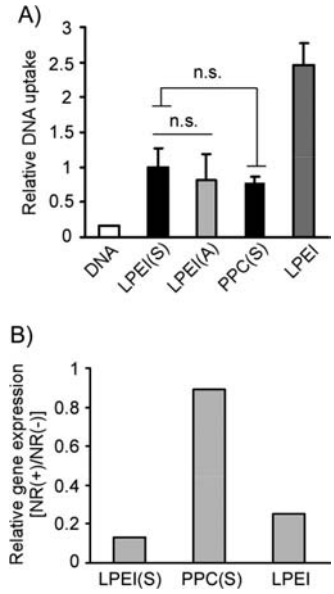

Figure 7. (A) Cellular uptake of polyplexes of fluorescein-labeled pDNA with each polymer. The pDNA uptake was determined 4 h after transfection (n = 3). (B) Relative transgene expression in the absence or presence of nigericin (NR), which is an inhibitor of endosomal acidification and inhibits the endosomal escape of the polyplexes (n = 4). N/P ratios of polyplex with LPEI–peptide conjugates (LPEI(S) and LPEI(A)), PPC(S), and LPEI were 10, 4, and 10, respectively. n.s.: not significant. Data are means ± SD.

will be explained by the weaker condensation ability of LPEI(S) than LPEI as observed in Figure 2. The above-mentioned lower expression in LPEI(S) than LPEI (Figure 6A) may also be attributable to this lower cellular uptake in LPEI(S) as shown in Figure 7A.

As described above, LPEI(A) showed a much lower expression than LPEI(S), indicating that after efficient escape from the endosome, the LPEI–peptide conjugates polyplexes are tolerant toward the exchange reaction with cytosolic macromolecules until the phosphorylation by PKCα. Thus, both the highly tolerant nature of the polyplexes and their quite sensitive transgene expression induced by PKCα are the origin of the all-or-none type response of the LPEI–peptide conjugates.

Cancer-Specific Gene Expression in Vivo. Finally, LPEI–peptide conjugates and PPCs were applied to transgene regulation in vivo by using model mice xenografted with the HepG2 cancer in subcutaneous tissue. A polyplex from each polymer was directly injected into the cancer tissue or normal subcutaneous tissue possessing a very low intracellular activity of PKCα (Figure S5, Supporting Information). We previously reported in vivo transfection in several types of xenografted cancers using PPC(S) polyplexes,[32−34] but we had not succeeded with HepG2 tissue, probably because of the low transfection efficiency of PPC(S) polyplexes. This result, reproduced again in parts A and B of Figure 8, showed quite a low expression level from the PPC(S) polyplex in the cancer. However, the LPEI(S) polyplex successfully exhibited a significant transgene expression in the cancer, comparable with that of the LPEI polyplex, while suppressing expression to a very low level in the normal skin tissue. The negative control LPEI(A) polyplex suppressed the transgene expression both in cancer and in skin tissues. Figure 8C summarizes the relative transgene expression in the cancer tissue and the normal skin tissue. LPEI(S) showed more than 20 times higher expression in cancer tissue than in normal skin, while the other polyplexes showed a relatively low level of expression. These results clearly demonstrate that LPEI(S) is a promising gene carrier that achieves both high transfection efficiency and cancer-specific transgene expression in vivo.

We did not inject the polyplex intravenously because the stability of the LPEI(S) polyplex is not enough in a blood flow. Figure S6 in the Supporting Information shows the luciferase expression in Balb/c mice after intravenous injection of the polyplexes. The LPEI polyplex showed high expression in lung, which is typically observed in previous reports.[46,47] However, the LPEI(S) polyplex showed no expression in any organs, indicating that the degradation of pDNA in a blood flow was due to the dissociation of the polyplex. Thus, additional modifications of LPEI(S) such as disulfide bonds and

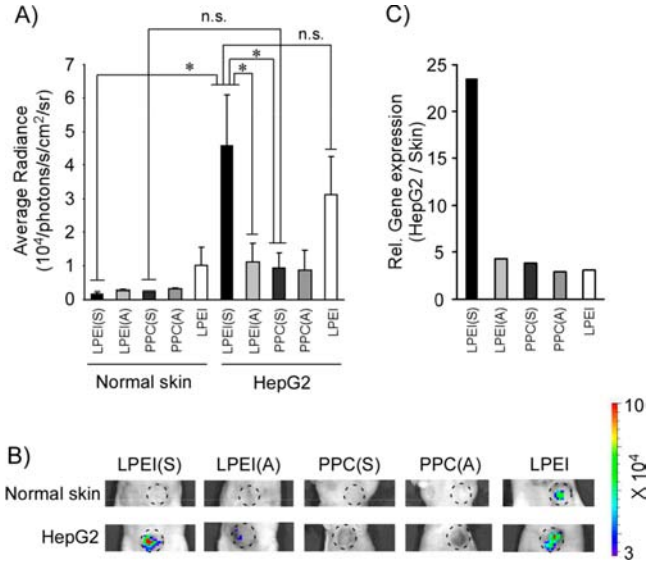

Figure 8. Transfection of various polyplexes into HepG2-xenografted mice. (A) Luciferase expression and (B) images after direct injection of polymer/pDNA polyplexes into HepG2 cancer tissues or normal subcutaneous tissues. The injection site of the polyplexes into normal skin and cancer tissues is indicated by black circles (n = 4). (C) Ratio of transgene expression in HepG2 cancer tissues to that in normal skin tissues. Asterisk: $P < 0.05$ indicates the significant difference in average radiance between groups. n.s.: not significant. Data are means ± SD.

hydrophobic interactions are needed to stabilize the resulting polyplex for intravenous injection.

■ CONCLUSION

We established straightforward synthesis of LPEI−peptide conjugates using click chemistry that is orthogonal to the reactivity of peptide side groups. The resultant conjugates showed all-or-none type response toward PKCα activity in transgene expression both in vitro and in vivo. In vitro studies for various cancer cell lines showed that a negative control LPEI(A) polyplex strongly suppressed transgene expression through binding with densely grafted cationic peptides. In contrast, LPEI(S) sharply responded to hyperactivated PKCα to efficiently express the transgene. LPEI(S) showed 20 to 400 times higher transgene expression than LPEI(A), depending on the PKCα activity of the examined cell lines. Because of its efficient endosomal escape and highly sensitive response to PKCα, the LPEI−peptide conjugate exhibited tumor-specific expression even in xenografted HepG2 tissue in which our previous carriers PPC showed negligible gene expression. Therefore, the LPEI−peptide conjugates are a promising carrier for cancer cell-specific gene therapy especially when toxic suicide genes are used. Our future efforts will be devoted to stabilizing the polyplexes of the LPEI−peptide conjugates in a blood flow for their intravenous administration. The stabilized polyplexes via disulfide bonds or hydrophobic interactions will be reported in our forthcoming paper.

■ METHODS

Materials. Rink Amide AM resin (200−400 mesh, amine density of 0.68 mmol/g), Fmoc-protected amino acids were purchased from Novabiochem (Darmstadt, Germany), 1-hydroxybenzotriazole hydrate (HOBt•H_2O), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIPEA), dichloromethane (DCM), 1-methyl-2-pyrrolidone (NMP), and piperidine were purchased from Watanabe Chemical (Hiroshima, Japan), and *N*,*N*-dimethylformamide (DMF) from Kanto Chemical (Tokyo, Japan). 5-Heynoic acid, sodium ascorbate, copper(II) sulfate pentahydrate, sodium azide, methacrylic acid, pyridine, dimethyl sulfoxide (DMSO), and ethanol were purchased from Wako Pure Chemicals (Osaka, Japan). 5-Chloro-1-pentyne and 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) were purchased from Tokyo Kasei Industry (Tokyo, Japan). Linear polyethylenimine (LPEI; M_w = 25 000) was purchased from Polysciences, Inc. (Warrington, PA, USA). According to the manufacture, M_w/M_n of precursor polymer, poly(2-ethyl-2-oxazoline), is 1.9 and LPEI contains 8 mol % of unhydrolyzed oxazoline unit. All reagents were used without further purification.

Synthesis of *N*-Terminus Azido Peptide. The peptide was synthesized by standard Fmoc-chemistry, using the Rink Amide AM resin (200−400 mesh, amine density of 0.68 mmol/g), DIPEA as a base, HOBt/HBTU as coupling reagents, and a 20% solution of piperidine in DMF for deprotection of Fmoc group. Presence of free amines was checked by the standard Kaiser (ninhydrin) test. Modification of the azide group at the N-terminus of the peptide was performed as follows: 3-Bromopropionic acid was substituted with the azide group by addition of 2 equiv of sodium azide for 2 days at RT in 1:1 DMF/DMSO, followed by addition of crude 3-azidopropionic acid to peptides for condensation with the N-terminus amino group of the peptide in the presence of coupling reagents. After competition of the peptide sequence, the resin was washed with DMSO, DMF, DCM, and methanol (each five times) and dried in vacuo overnight. Cleavage of the peptide from the resin and side chain deprotection was performed by the treatment of a mixture of TFA/ethanedithiol/triisopropylsilane/water (94/2.5/2.5/1) for 90 min at RT. The material was filtered and washed with a minimum amount of cleavage reagent described above. The peptide solution was added dropwise into an excess volume of diethyl ether to precipitate peptide, followed by centrifugation (5000g, 4 °C) to collect precipitate. This cycle was performed three times then the precipitate was dried in vacuo overnight. The crude peptides obtained were purified by reverse-phase liquid chromatography equipped with an electrospray mass spectrometer for the detector [Micromass Platform II connected to Waters Alliance HPLC system with a Phenomenex LUNA C18 column (2.1 × 50 mm)], using a linear gradient at a flow ratio of 20 mL/min with an acetonitrile/water mobile phase containing 0.1% TFA.

Synthesis of Alkyne-Functionalized LPEI (LPEI−Pentyne). LPEI (0.3 g; 1.2 μmol; 7.0 mmol as secondary amine) was dissolved in dry DMSO (30 mL) followed by addition of pyridine (1.1 g; 14.0 mmol), DBU (0.32 g; 2.1 mmol), and 5-chloro-1-pentyne (0.72 g; 7.0 mmol). The mixture was stirred overnight under an N_2 atmosphere at 50 °C. After the reaction, DMSO was partially evaporated under reduced pressure. The residue was diluted in methanol and dialyzed against 3 L of methanol solution containing 0.05 N HCl (changed 3 times) and 3 L of water (changed 5 times) by using a dialysis membrane bag (MW cut off, 3 500). After dialysis, the mixture was partially evaporated under reduced pressure and then freeze-dried to obtain a yellow powder. The content of alkyne groups in LPEI−pentyne was determined as 20.4 mol % from ^{1}H NMR spectra (Figure S1, Supporting Information).

Synthesis of LPEI−Peptide Conjugate. LPEI−pentyne (3.3 mg, 7.3 μmol as alkyne), *N*-azido-peptide [N_3-$(CH_2)_2$-CO-FKKQGSFAKKK-NH_2; 5.0 mg; 3.6 μmol], copper(II) sulfate pentahydrate (1.8 mg; 7.2 μmol), and sodium ascorbate (28.5 mg; 144 μmol) were dissolved in 808 μL of water/ethanol (1/1 v/v) and the mixture was stirred at RT for 1 day. After the reaction, crude product was dialyzed against 3 L of 0.05 N HCl (changed twice) and 3 L of water (changed 6 times), using a dialysis membrane bag (MW cut off, 3 500), followed by freeze-drying to obtain a yellow-to-brown powder. The peptide content in LPEI(S) was calculated from trinitrobenzenesulfonic acid (TNBS) assay. LPEI(A) was synthesized by the same procedure.

Ethidium Bromide (EtBr) Exclusion Assay. Polymer/pDNA polyplexes were prepared at various *N*/*P* ratios with EtBr in sterilized water. After incubation of the mixture for 20 min, the final

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concentration of pDNA and EtBr was adjusted to 50 and 12.5 μg/mL with 10 mM HEPES−NaOH buffer (pH 7.3), respectively. Fluorescence measurements of each sample were performed at 25 °C by the multilabel counter ARVO (Wallac Incorporated, Turku, Finland) as previously described. Excitation and emission wavelengths were 531 and 590 nm, respectively. The relative fluorescence intensity (RFI) was determined by using the following equation: RFI = $(F_{obs} - F_e)/(F_0 - F_e)$, where F_{obs}, F_e, and F_0 are the fluorescence intensities of the polymer/pDNA polyplex at each N/P ratio, EtBr alone, and pDNA plus EtBr without polymer, respectively.

Dynamic Light Scattering (DLS). Fifty microliters of pDNA (0.1 mg/mL) solution and 50 μL of polymer solution were mixed and allowed to stand for 20 min at RT. The final volume of the polyplex dispersion was adjusted to 1 mL by 10 mM HEPES buffer (pH 7.3). Size and ζ-potential of polyplexes were determined by using a Zetasizer Nano instrument (Malvern Instruments Limited, Malvern, UK).

Cell Culture. HepG2 (human hepatoma), U87-MG (human glioma), B16 melanoma (mouse melanoma), and Neuro2A (N2A; mouse neuroblastoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen Co., Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco). Cells were harvested in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

Transfection Studies. Cells were seeded on 48-well plates at an initial density of 25 000 cells/well in DMEM containing 10% FBS and grown for 2 days. Polyplexes of pDNA with each polymer at various N/P ratios were prepared by simply mixing them in sterilized water for 20 min. The final concentration of pDNA was adjusted to 2.5 μg/mL by the Opti-MEM (Gibco). After washing the wells with phosphate buffered saline (PBS), 200 μL of polyplex solutions was applied to each well. After incubation for 4 h, medium was exchanged with DMEM containing 10% FBS, followed by further incubation for designated times. In the case of nigericin (NR) treatment, 1 μM of NR and polyplex solution were co-added to the well. Luciferase expression was evaluated by using a luciferase assay kit and a luminometer as described previously.

Cellular Uptake of pDNA/Polymer Polyplex. Measurement of cellular uptake of polymer/pDNA polyplexes were performed by using the fluorescein-labeled pDNA. Chemical labeling of the fluorescein to pDNA was carried out by using a LabelIT Fluorescein Labeling Kit (Mirus, Madison, USA) according to the manufacturer's protocol. Formation of polyplex and transfection methods were performed as described above. Four hours after transfection of polyplex, each plate well was washed twice with 150 μL of PBS and cells were lysed with 100 μL of lysis buffer (pH 7.5, 20 mM Tris-HCl buffer, 0.05% TritonX-100 and 2 mM EDTA). Fifty microliters of each lysate were moved to blacked-bottomed 96-well plates, and fluorescence intensity was measured at an excitation and emission filter with wavelengths of 490 and 535 nm, respectively, by the multilabel counter ARVO. The relative uptake was determined by using the following equation: relative uptake = $(F_{obs} - F_e)/(F_{PPC(S)} - F_e)$, where F_{obs}, F_e, and $F_{PPC(S)}$ are the fluorescence intensity of polymer/pDNA polyplexes, sterilized water, and the PPC(S)/pDNA polyplex, respectively.

Western Blotting. For lysate preparation from normal subcutaneous and HepG2 tissues, samples were excised from mice, weighed, and homogenized in 1 mL of buffer (20 mM Tris-HCl, pH 7.5, 250 mM sucrose and Complete protease inhibitor cocktail). The homogenate was centrifuged at 900 × g at 4 °C for 10 min and the supernatant was removed. After washing with 1 mL of buffer and recentrifuging, 1 mL of buffer was added into the precipitate. Samples were sonicated for 30 s, and then centrifuged at 5 000 × g at 4 °C for 15 min, and the resulting supernatant was immunoblotted with anti-PKCα serum (Cell Signaling, MA, USA), anti-phosphoPKCα (Ser657) serum (Upstate, MA, USA), or antiactin serum (Santa Cruz Biotechnology, CA, USA), and the reacting proteins were visualized by chemiluminescence.

Animal Studies. Animal studies were performed in accordance with the Guidelines for Animal Experiments of Kyushu University. Male 4-week-old BALB/c nude mice were used in this study. Mice were inoculated with a dorsal, subcutaneous injection of 1×10^7 cells in 100 μL of Matrigel (BD Biosciences, Bedford, MA, USA) per animal. Tumors were allowed to grow to a mean diameter of approximately 8 mm. Introduction of 100 μL of various polyplexes [DNA dose, 5 μg/mouse; N/P = 10 for LPEI(S), LPEI(A), and LPEI or N/P = 4 for PPC(S) and PPC(A)] into cancer or subcutaneous tissue was performed by a direct injection. After 24 h, mice were anesthetized and injected intraperitoneally with 200 μL of 15 mg/mL D-luciferin potassium salt (Promega, Madinson, WI, USA) in Ringer's solution. Images were obtained with use of a cooled IVIS CCD camera (Xenogen, Alameda, CA, USA) and analyzed with Living Image software.

Statistical Analyses. Student t-tests were used to analyze differences among means of groups. P values <0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

Characterization of polymer/pDNA polyplexes, cell studies, and Western-blotting of PKCα contained in cancer and normal tissues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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