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RESEARCH PAPER

Poly-L-arginine and Dextran Sulfate-Based Nanocomplex for Epidermal Growth Factor Receptor (EGFR) siRNA Delivery: Its Application for Head and Neck Cancer Treatment

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ABSTRACT

Purpose A poly-L-arginine (PLR) and dextran sulfate (DEX)-based nano-sized polyelectrolyte complex (nanocomplex) was developed for epidermal growth factor receptor (EGFR) siRNA delivery for the treatment of head and neck cancer.

Methods PLR and DEX-based nanocomplex including EGFR siRNA was prepared and characterized. *In vitro* cellular uptake efficiency and EGFR gene silencing effect of nanocomplex including EGFR siRNA were evaluated in Hep-2 and FaDu cells. Its *in vivo* anti-tumor efficacy was also assessed in FaDu tumor xenografted mouse model.

Results The weight ratio of polymer:RNA was 15:1 and a nanocomplex system consisting of <200 nm in mean diameter and a positive surface charge was prepared. According to the results of confocal laser scanning microscopy (CLSM) and flow cytometry analyses, the PLR-DEX complex exhibited the best cellular uptake efficiency of EGFR siRNA in Hep-2 and FaDu cells, which led to the highest EGFR gene silencing efficiency in both cell lines. PLR-DEX/EGFR siRNA complex exhibited efficient tumor growth inhibition and EGFR silencing effect in a tumor xenografted mouse model.

Conclusion PLR and DEX-based nanocomplex containing EGFR siRNA was successfully developed. The new formulation was effective in EGFR gene silencing and tumor growth inhibition in head and neck cancer cells.

KEY WORDS dextran sulfate · EGFR siRNA · head and neck cancer · nanocomplex · poly-L-arginine

INTRODUCTION

RNA interference has recently been introduced as an attractive strategy for cancer therapy (1–3). However, although siRNA-related biological functions occur primarily in the cytoplasm, its high molecular weight and negative charge can hamper its intracellular uptake. Moreover, in addition to membrane impermeability and later endocytosis, *in vivo* siRNA delivery can also encounter obstacles of rapid hydrolysis by serum nucleases (4,5). Lipoplexes have been investigated as a siRNA delivery system and Lipofectamine 2000 has been widely used for *in vitro* gene transfection (6). However, this approach has some drawbacks if applied *in vivo*, including excessive particle size, low stability, and insufficient siRNA entrapment (7); thus, the siRNA can be degraded by enzymatic and physical attack in body fluids. Delivery systems using nanoparticles have been investigated to increase the stability of siRNA and to facilitate its introduction into cells (8,9). An attractive delivery system is that of nano-sized polyelectrolyte complexes composed of biocompatible polymers (10,11). This kind of systems can be formed by electrostatic interactions and can be easily prepared. Because no heat or organic solvent is required, the siRNA can be maintained intact during the preparation process. The *in vivo* performance of these complex systems containing siRNA has been demonstrated (10,11).

Head and neck cancer is a generic term for different tumor types in the oral cavity, nose, throat, sinuses, and salivary glands. Patients with early stage cancer are typically treated with surgery and/or radiotherapy. However, patients with advanced-stage disease have a 5-year survival of less than 10% and recurrent/metastatic patients have a

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median survival of no longer than 1 year (12,13). The most commonly used therapeutic agents are platinum-based drugs (cisplatin or carboplatin) and their combination with taxanes or 5-fluorouracil. The response rate to platinum-based therapeutics is <30% and the survival benefits for recurrent/metastatic patients may be as little as 10 weeks (14). To improve the response rate and survival period, various therapeutic approaches have been tried. Several molecular targets with high selectivity for head and neck cancer have been investigated. Among these, epidermal growth factor receptor (EGFR) is considered to be an attractive target because the activation of downstream signaling might consequently enhance anti-apoptosis, invasiveness, and proliferation of squamous cell carcinoma of the head and neck (SCCHN) (15). EGFR, also known as HER1 or c-erbB-1, is a 170-kDa glycoprotein composed of an extracellular, transmembrane, and intracellular domain with tyrosine kinase function. In particular, when EGFR and its ligand, transforming growth factor-alpha (TGF-α), were overexpressed in a SCCHN specimen, there was a 70- and 5-fold increase in EGFR and TGF-α mRNA, respectively (16).

To inhibit EGFR signal transduction, various approaches have been developed. EGFR monoclonal antibodies (mAbs) may prevent the dimerization of the receptor by binding to it. Cetuximab (Erbitux), a chimeric (mouse/human) monoclonal antibody that binds to EGFR, was approved by the U.S. Food & Drug Administration (FDA) for colorectal, lung, and head and neck cancer treatment. Small-molecule tyrosine kinase inhibitors impede EGFR signal propagation by blocking tyrosine kinase activation at the adenosine triphosphate (ATP) binding site; some drugs (*e.g.*, gefinitib, erlotinib) are approved by the FDA and others are undergoing clinical trials. Gene therapy using antisense oligonucleotides or siRNA for EGFR has also been investigated for the treatment of head and neck cancer (17,18). However, to date, there has been no report of an effective intratumoral siRNA delivery system for head and neck cancer.

In this investigation, nanocomplex, composed of poly-L-arginine (PLR) and dextran sulfate (DEX), was developed for EGFR siRNA delivery for the treatment of head and neck cancer. With negatively charged siRNA, DEX, an anionic polymer, was used to prepare nanocomplex with PLR as a cationic polymer. Although polyelectrolyte microcapsules composed of PLR and DEX have been reported as an antigen delivery system (19), their use as a siRNA delivery system has not been investigated. PLR, composed of arginine, can play a role as a membrane-penetrating peptide and thus can be an important motif for the intercellular delivery of macromolecules (20). DEX, as a biodegradable and biocompatible anionic polymer, contains branched anhydroglucose units with about 2.3 sulfate groups per glucosyl residue (21). Since polyanions have been reported to make compact siRNA complexes (22,23), addition of DEX in the formulation could further reduce their particle size. Herein, we report the development and characterization of a nanocomplex system composed of PLR and DEX containing EGFR siRNA. Its cellular uptake and gene silencing effects were evaluated in head and neck cancer cell lines, and the *in vivo* anti-tumor efficacy was also assessed in a tumor-bearing mouse model.

MATERIALS AND METHODS

Materials

EGFR siRNA, negative control siRNA (scrambled siRNA, scRNA), and FITC-labeled EGFR siRNA were purchased from Bioneer Inc. (Daejon, Korea). Poly-L-arginine (PLR, molecular weight: 15–70 kDa) and dextran sulfate (DEX, molecular weight: 6.5–10 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS), and other cell culture reagents were obtained from Gibco BRL (Grand Island, NY). All the other chemicals and reagents were of analytical grade.

Preparation of Nanocomplex System

PLR/siRNA polyplexes were prepared by mixing various amounts of PLR (0.165-16.5 μg) and a fixed amount of EGFR siRNA (0.66 μg); the weight ratio ranged from 0.25 to 25. PLR-DEX/siRNA polyplexes were formed by blending several amounts of PLR-DEX (0.165–16.5 μg) with EGFR siRNA (0.66 μg) as the PLR:DEX weight ratio (10:1) was fixed. Polymers and siRNA were solubilized in diethylpyrocarbonate (DEPC)-treated distilled water (DW) and polyplexes were stabilized for 20 min at room temperature before use.

Characterization of EGFR siRNA-Loaded Nanocomplex

The binding of siRNA and polymer was confirmed by 2% agarose gel electrophoresis. Although the ratio between PLR and DEX was fixed (10:1), samples with different weight ratios between polymer and siRNA were loaded onto the gel. Gel electrophoresis was conducted at 100 V for 30 min using Tris-borate-EDTA (pH 8.3; TBE) buffer. The gel was stained using GelRed (Biotium, Inc., Hayward, CA) and observed under a UV illuminator.

Particle size, polydispersity, and zeta potential were measured using a light scattering spectrophotometer ELS-Z (Otsuka Electronics, Japan). siRNA stock solution (25 μl of 100 μM) with polymers was diluted with double distilled

water (DDW) and analyzed according to the manufacturer's instructions.

The morphology of the PLR-DEX/siRNA complex was observed by TEM analysis. PLR-DEX/siRNA (20 μl) was diluted in DDW and stained with 2% (w/v) of phosphotungstic acid (PTA). Samples were placed on copper grids with films and dried in air for 10 min before TEM observation (JEM 1010; JEOL, Japan).

In Vitro Cytotoxicity

Hep-2 and FaDu cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Hep-2 cells were cultured with DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, whereas FaDu cells were cultured with MEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a 5% CO_2 atmosphere and 95% relative humidity at 37°C. After Hep-2 and FaDu cells reached 70–80% confluency, they were trypsinized and seeded in a 96-well plate at a density of 1.0×10^4 per well. After 24 h of incubation, the cell culture media were removed. After 12 and 24 h incubation of PLR/scRNA (15:1 weight ratio of polymer:RNA) and PLR-DEX/scRNA (5:1 to 25:1 weight ratio of polymer:RNA and 10:1 weight ratio of PLR:DEX) in those cells at 37°C, 5% CO_2 and 95% relative humidity, the cytotoxicity was measured by treating with MTS-based CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay Reagent (Promega Corp., WI, USA) at 37°C for 4 h according to the manufacturer's protocol. The absorbance at 490 nm was measured using an EMax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA).

RNase Protection Assay

RNaseONE ribonuclease (Promega, Madison, WI) was used to measure the stability of siRNA and siRNA in nanocomplex against enzymatic degradation. Polymers and siRNA (1.33 μg) were solubilized in DEPC-treated DW. PLR/siRNA (15:1 weight ratio), PLR-DEX/siRNA (10:1 and 15:1 weight ratios for PLR:DEX and PLR-DEX:siRNA, respectivley), and Lipofectamine 2000/siRNA were stabilized for 20 min at room temperature. They were then incubated in 10 μl of reaction buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM sodium acetate) with 5 U of RNase for 15, 30, and 60 min. Then, 50 μg of heparin sodium was added to the reaction and incubated for 10 min at room temperature. Gel electrophoresis was performed using 2% agarose to confirm the degradation of RNA.

In Vitro Cellular Uptake

After Hep-2 and FaDu cells reached 70–80% confluency, the cells were trypsinized and seeded on culture slides (BD Falcon, Bedford, MA) at a density of 4.0×10^4 cells per well (1.7 cm^2 of surface area per well, four-chamber slides) and incubated for 24 h at 37°C for confocal laser scanning microscopy (CLSM) study. Next, 20 nM FITC-siRNA complex with or without 2 μl of Lipofectamine 2000 (1 mg/ml) or polymer was added and incubated for 2 h at 37°C. After 2 h of incubation, all reagents were removed. Cells were washed with phosphate-buffered saline (PBS, pH 7.4) for 15 min at least three times and fixed with 4% formaldehyde solution for 15 min, and then dried completely. VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; H-1200; Vector Laboratories, Inc. CA) was added to prevent fading. The cells were observed using confocal laser scanning microscopy (LSM 510; Carl-Zeiss, Thornwood, NY) using an excitation wavelength of 543 nm. The magnification ratio was × 400.

For flow cytometry analysis, after Hep-2 and FaDu cells reached 70–80% confluency, the cells were detached and seeded in a 6-well plate at a density of 6×10^5 cells per well and incubated overnight. Culture media were removed and 20 nM FITC-siRNA with polymer was added and incubated for 2 h. After incubation, all reagents were removed and cells were washed with PBS at least three times. After washing, cells were trypsinized and the supernatant was carefully removed. PBS containing 2% (v/v) FBS was added to resuspend the cell pellet. The FITC-siRNA contained in the cells was analyzed using a FACSCalibur fluorescence-activated cell sorter (FACS) equipped with CELLQuest software (Becton Dickinson Biosciences, San Jose, CA).

In Vitro Gene Silencing Study by RT-PCR

Hep-2 and FaDu cells were seeded on 12-well plates at a density of 4.0×10^5 cells per well and incubated for 24 h, followed by treatment with 100 nM EGFR siRNA or scRNA for 24 h. The sequence of EGFR siRNA was 5′-GAUCCA CAGGAACUGGAUAdTdT-3′ (sense) and 5′-UAUCCA GUUCCUGUGGAUCdTdT-3′ (antisense). The sequence of the negative siRNA (scRNA) was 5′-CCUACGCCAC CAAUUUCGUdTdT-3′ (sense) and 5′-ACGAAAUUG GUGGCGUAGGdTdT-3′ (antisense). The primer sequence of the EGFR mRNA (P187403, Bioneer Corp., Korea) was not disclosed by the manufacturer, but its size was 155 bp. Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were also prepared (190 bp; Bioneer Corp.): 5′-TGGTATCGTGGAAGGACTCATGAC-3′ (forward) and 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ (reverse). The weight ratio of PLR:siRNA was 15:1 and that of PLR:DEX and PLR-DEX:siRNA was fixed at 10:1 and 15:1, respectively. Total RNA was isolated using the RNeasy plus mini kit (Qiagen) and AccuPower CycleScript RT premix (Bioneer Corp.) was used for reverse transcription (RT). For PCR, AccuPower HotStart PCR premix (Bioneer Corp.) was

used under the following conditions: denaturation (95°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 1 min).PCR products were loaded onto a 2% agarose gel and observed using a UV illuminator. The EGFR mRNA expression level was normalized to that of the GAPDH gene and each relative expression level was compared using the ImageJ program (ver. 1.43r).

Apoptosis Assay

The apoptotic effect of EGFR siRNA with or without polymer was investigated in Hep-2 and FaDu cells. Both cell lines were seeded in 6-well plates at a density of 6×10^5 cells per well and incubated overnight. Next, 100 nM EGFR siRNA and negative control siRNA (scRNA) with or without PLR and PLR-DEX were added and incubated for 24 h. Cells were washed with PBS and harvested. After centrifugation (16,100×*g*, 5 min) cells were resuspended in reaction buffer. Using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA), cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's protocol. Both cell types were analyzed by FACSCalibur fluorescence-activated cell sorter equipped with CELLQuest software (BD Biosciences).

In Vivo Antitumor Efficacy

A female BALB/c nude mouse (5 weeks old; Charles River, Washington, MA) was used to prepare the tumor xenografted mouse model. Mice were maintained in a light-controlled room kept at a temperature of 22±2°C and a relative humidity of 55±5% (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University, Korea). The experimental protocols involving animal study were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University. A FaDu cell suspension (3×10^6 cells in 0.1 ml) was injected subcutaneously in the right back. Tumor treatment was started 14 days after injection, when the tumor volume reached about 50–100 mm^3. Tumor size was measured with Vernier calipers and tumor volume (mm^3) was calculated by the formula $V = 0.5 \times \text{longest diameter} \times \text{shortest diameter}^2$. EGFR siRNA (6.65 μg) was injected intratumorally to each mouse three times for 5 days. Groups were divided as follows: control, naked siRNA, PLR/siRNA (15:1 weight ratio of polymer:RNA) and PLR-DEX/siRNA (15:1 weight ratio of polymer:RNA and 10:1 weight ratio of PLR:DEX). Tumor volumes and body weight were measured for 20 days. For immunohistochemical staining, dissected tumors were fixed with 4% (v/v) formaldehyde solution for 1 day and 6-μm tumor sections were deparaffinized and hydrated with ethanol. EGFR monoclonal antibody (1:200 dilution; Lab Vision/Thermo Fisher Scientific Inc., Fremont, CA) was added to the tumor sections and then the secondary horseradish peroxidase-labeled antibody was applied. The chromogen diaminobenzene (DAB) was incubated for color development in immunohistochemical staining. Frozen sectioned tumors were also stained with hematoxylin and eosin (H&E) according to a standard procedure.

Statistical Analysis

All experiments in this study were performed at least three times and the data are expressed as the mean ± standard deviation (S.D.). Statistical analysis was performed using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Preparation and Characterization of Nanocomplex Containing EGFR siRNA

A gel retardation assay was used to evaluate the formation of PLR-DEX/siRNA by electrostatic interaction according to various weight ratios of PLR-DEX:siRNA (from 0.25:1 to 10:1). As shown in Fig. 1, the siRNA band weakened as the polymer content increased. The siRNA band was observed up to a weight ratio of 1:1 PLR-DEX:siRNA, and disappeared at ratios greater than 2.5:1. The disappearance of the siRNA band indicated the neutralization of the negative charge of RNA and DEX (anionic polymer) and the positive charge of PLR (cationic polymer) by forming nano-sized complex. Although the gel retardation assay results for PLR/siRNA are not shown, it was observed that the PLR/siRNA complex was also formed at PLR:siRNA weight ratios greater than 2.5:1. Together, these data

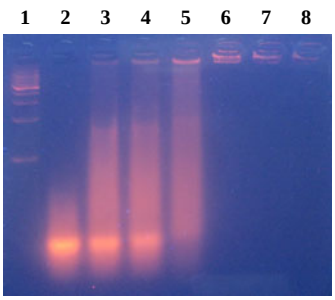


Fig. 1 The image of gel retardation assay with PLR-DEX/siRNA complex. PLR-DEX was complexed with siRNA at several weight ratios and then they were electrophoresed on the agarose gel. Weight ratio between PLR and DEX was fixed as 10:1 and weight ratio between PLR-DEX and siRNA was varied.

indicated that PLR or PLR-DEX complex with siRNA was formed at weight ratios over 2.5:1, to be used as a siRNA delivery system.

To determine the optimal ratio for preparing PLR-DEX/siRNA, the mean diameter, polydispersity index, and zeta potential were measured in the range of 5:1 to 25:1 weight ratio. The mean diameter of PLR-DEX/siRNA decreased as the weight ratio increased (Table I). A nano-sized complex (<200 nm) was formed with more than 15:1 weight ratio. As the PLR-DEX:siRNA ratio increased, the zeta potential also increased (Table I). As the ratio of PLR-DEX:siRNA increased, the amount of DEX, used as an anionic polymer, also increased. However, the ratio between PLR:DEX was fixed at 10:1; thus, it seemed that the relatively higher increase in PLR amount contributed to the increase of zeta potential. At about 15:1 weight ratio, positively charged nanoparticles for intracellular uptake were formed. The morphological shape was observed by TEM imaging. Nanocomplexes containing EGFR siRNA with similar size ranges presented in Table I were stained and observed (Fig. 2).

In Vitro Cytotoxicity

The cytotoxicity of naked scRNA, Lipofectamine2000 + scRNA, PLR/scRNA and PLR-DEX/scRNA was assessed over 12 and 24 h using the MTS-based assay (Fig. 3). To evaluate cytotoxicity of the polymeric components, scRNA-loaded nanocomplex systems were utilized in this study. The weight ratio of PLR:scRNA was 15:1 and those of PLR:DEX and PLR-DEX:scRNA were 10:1 and 5:1–25:1, respectively. No significant difference in polymer cytotoxicity was observed regardless of the cell line and incubation period used. Moreover, no serious cytotoxicity was detected in all polymer concentration used in the current study. The optimal weight ratio of PLR-DEX to RNA was determined as 15:1 based on the mean diameter, zeta potential, and cytotoxicity of nanocomplex. This ratio was used in the formulations for further studies.

Table I Size Analysis and Zeta Potential Measurement of PLR-DEX/siRNA Complex

Polymer:siRNA ratio[a]	Size analysis		Zeta potential (mV)
	Mean diameter (nm)	Polydispersity	
5:1	364.03 ± 37.96	0.26 ± 0.02	−1.41 ± 1.40
10:1	279.83 ± 17.86	0.25 ± 0.06	0.61 ± 0.83
15:1	183.90 ± 11.42	0.21 ± 0.03	8.06 ± 1.86
20:1	193.03 ± 8.69	0.27 ± 0.02	7.77 ± 2.69
25:1	132.40 ± 0.53	0.24 ± 0.01	9.30 ± 0.79

[a] The weight ratio between PLR and DEX was fixed as 10:1

RNase Protection Assay

To evaluate the protection of siRNA entrapped in nanocomplex from RNase, siRNA alone or with nanocomplex was incubated for 60 min, followed by gel electrophoresis. As shown in Fig. 4a, the naked siRNA was degraded completely by nucleases within 15 min. Lipofectamine 2000/siRNA complex partially protected siRNA from degradation, and the siRNA band intensity weakened over time (Fig. 4b). In contrast, siRNA bands in PLR and PLR-DEX complexes (Fig. 4c and d) were still intense after 1 h of incubation. This indicated that the siRNA incorporated in PLR and PLR-DEX complexes was not degraded by nuclease attack. These results indicate that nanocomplex could contribute to siRNA stability when it's in contact with biological fluids in the body.

In Vitro Cellular Uptake

In Hep-2 and FaDu cell lines, the cellular uptake efficiency of the prepared nanocomplex system with siRNA was measured by CLSM and flow cytometry (Fig. 5). The weight ratio (polymer:RNA) was set at 15:1, for both PLR/siRNA and PLR-DEX/siRNA groups, with incubation for 24 h. FITC-labeled siRNA was used in the cellular uptake studies and cell nuclei were stained with DAPI (blue color). Cellular uptake of siRNA occurs by its binding to the cellular membrane and a subsequent internalization process. As shown in Fig. 5a, naked FITC-siRNA was hardly taken up into the cell. Due to electrostatic repulsion between the strong negative charge of phosphate groups of RNA and the negatively charged cellular membrane, it is difficult for naked siRNA to penetrate the cellular membrane without a cationic carrier. Although Lipofectamine 2000 (a common *in vitro* transfection reagent) was also used as a control, FITC-siRNA was partially localized in the cytoplasm of the cell. FITC-siRNA delivered with PLR-DEX was distributed more uniformly in the perinuclear region of the cell than the PLR/siRNA complex. In our study, PLR-DEX/siRNA was localized primarily in the perinuclear region and no fluorescence signal was observed in the nucleus of the cell. FITC intensity (green color) seldom overlapped with the blue color of DAPI staining of the cell nucleus region. This observation has also been reported in the intracellular uptake study of arginine-based siRNA delivery systems (9,24).

The intracellular uptake efficiency of siRNA with nanocomplex was analyzed quantitatively by flow cytometry (Fig. 5b). It was evaluated by the percentage of cell number included in the M1 region, where almost no fluorescent

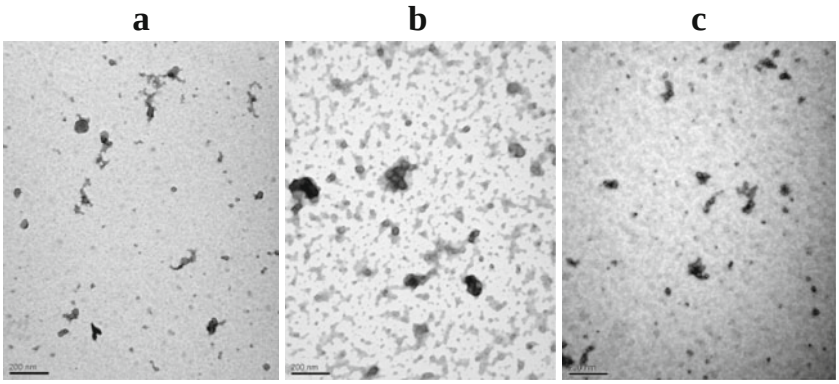


Fig. 2 Morphological shapes of PLR-DEX/siRNA complex observed by TEM. Weight ratio between PLR-DEX and siRNA was varied (**a**) 5:1, (**b**) 15:1 and (**c**) 25:1. The length of bar is 200 nm.

signal was detected in the control (no treatment) group. The representative percentage values of control, naked siRNA, Lipofectamine 2000/siRNA, PLR/siRNA, and PLR-DEX/siRNA groups were as follows: 0.88, 1.70, 40.60, 71.33, and 98.23% in Hep-2 cells and 0.24, 0.66, 48.83, 60.48, and 88.79% in FaDu cells, respectively. Thus, it can be concluded that the intracellular uptake efficiency of siRNA measured by flow cytometry in both cell lines followed the order PLR-DEX/siRNA > PLR/siRNA > Lipofectamine 2000/siRNA > naked siRNA. Cellular uptake efficiencies detected by flow cytometry were also largely consistent with the results in the CLSM study (Fig. 5a). The higher intracellular uptake efficiency of the PLR-DEX complex resulted from its positive surface charge, as reflected in the zeta potential measurement (Table I), and its electrostatic interaction with the cellular membrane. The PLR-DEX complex exhibited higher cellular uptake efficiency of siRNA than the PLR/siRNA group and this is thought to be caused by the difference in particle size between the two complexes. In this investigation, the mean diameter of the PLR/siRNA complex was 634.40 ± 27.78 nm and its polydispersity index was 0.30 ± 0.03. The particle size of the PLR-DEX/siRNA complex (Table I) was smaller than that of the PLR/siRNA complex ($P < 0.05$) and it is assumed that smaller particles were introduced more efficiently into the cell. Previously, it has been reported that the size of polyelectrolyte complex was the most critical factor in endocytosis and its intracellular fate (25). The PLR-DEX/siRNA complex with <200 nm in mean diameter exhibited the most efficient cellular uptake in both cell lines, indicating that it may be a candidate for the internalization and gene silencing of siRNA.

Gene Silencing

The cellular trafficking ability of PLR-DEX/siRNA has been established in previous cellular uptake studies. Based on the results of cellular uptake studies, EGFR gene silencing was evaluated in Hep-2 and FaDu cells. Hep-2 and FaDu cells originated from the epidermoid carcinoma tissue of the larynx and carcinoma of the pharynx, respectively. It is known that the EGFR gene is stably expressed in Hep-2 and FaDu cells (26,27) and its signaling pathway and function have been investigated. Thus, these two cancer cells were used for the EGFR gene silencing test. As shown in Fig. 6, the gene silencing effect of PLR-DEX was compared with that of the naked siRNA, naked scRNA, Lipofectamine 2000/siRNA, and PLR/siRNA complex groups. EGFR siRNA (100 nM) was incubated alone or with carrier for 24 h. Off-target silencing effects of the scRNA (Fig 6) and that of the polymeric nanocarrier with scRNA were not observed in both cell lines (Supplementary Fig. 1). However, the incubation of siRNA with Lipofectamine 2000, PLR, or PLR-DEX resulted in a significantly improved gene silencing effect ($P < 0.05$) compared with naked siRNA. Moreover, Lipofectamine 2000, a lipoplex-based commercial transfection reagent, did not exhibit a remarkable gene silencing effect compared with the PLR and PLR-DEX complexes. The percentages of gene silencing efficiency of PLR-DEX/siRNA in Hep-2 and FaDu cells were 95.2 and 81.7%, respectively (Fig. 6b). The EGFR gene silencing efficiency was ranked in the order PLR-DEX/siRNA > PLR/siRNA > Lipofectamine 2000/siRNA > naked siRNA. Results of the *in vitro* EGFR gene silencing tests where higher gene knockdown efficiency was observed for the PLR-DEX group against the PLR group ($P < 0.05$) coincided with those of the cellular uptake study.

Apoptosis Assay

It is known that EGFR down-signaling is related to apoptosis, cell proliferation, and metastasis in head and neck cancer cells. Anti-cancer effects of EGFR siRNA in SCCHN cell lines, such as HSC-2 and SAS cells, have been reported (18). Although the cell line was different from those used in this investigation, treatment with EGFR siRNA inhibited tumor cell growth over 1 week, enhanced the tumor cell growth inhibition effect of anti-cancer agents, increased the apoptotic effect in combination with chemotherapeutic agents, and

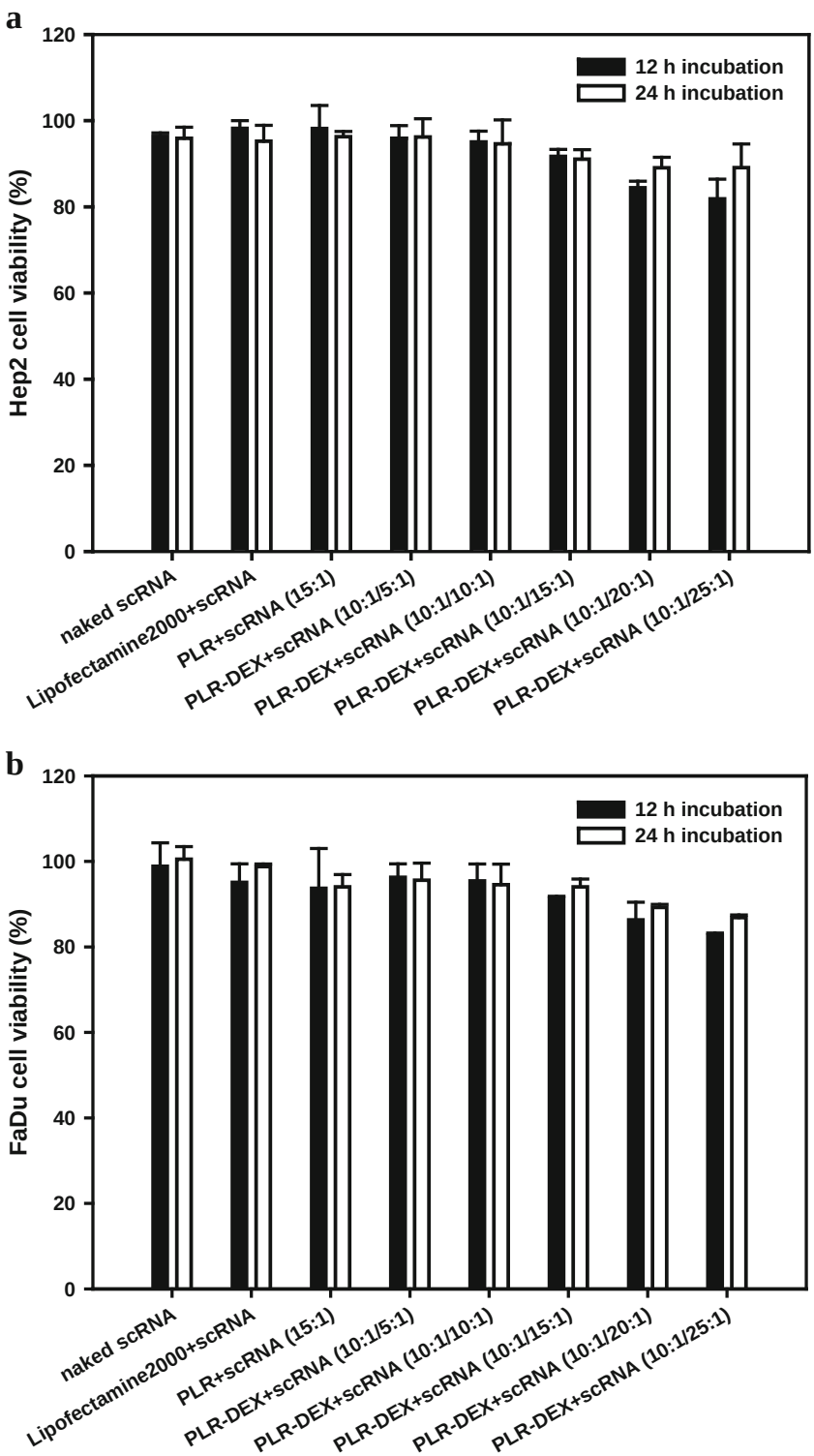


Fig. 3 *In vitro* cytotoxicity test in (**a**) Hep-2 and (**b**) FaDu cells. Naked scRNA, Lipofectamine2000 + scRNA, PLR/scRNA and PLR-DEX/scRNA (100 nM scRNA concentration) were incubated for 12 and 24 h after which the cell viability was measured by MTS assay (n=3). Cell viability (%) is presented as the ratio to the control (no treatment) group. The weight ratio of PLR:DEX was fixed as 10:1 while that of PLR-DEX:siRNA was varied from 5:1 to 25:1.

enhanced its antitumor effect in a xenograft model in combination with cisplatin. In our study, the apoptotic effect of EGFR siRNA with or without nanocomplex, as a method for demonstrating its anti-tumor effects, was measured. EGFR siRNA-treated cancer cells were double-stained with Annexin V-FITC and PI for the determination of apoptosis in each cell line. As illustrated in Fig. 7, the upper right panel and lower right panel indicate late apoptotic/necrotic and early apoptotic cells, respectively (28). The sum of the population percentage in the right panel, indicating the

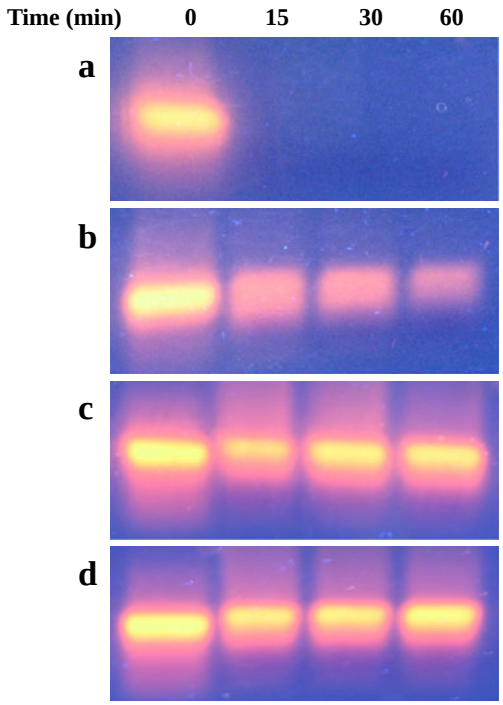


Fig. 4 RNase protection assay of (**a**) naked siRNA, (**b**) Lipofectamine 2000/siRNA, (**c**) PLR/siRNA (15:1 weight ratio), and (**d**) PLR-DEX/siRNA (Weight ratios for PLR:DEX and PLR-DEX:siRNA were 10:1 and 15:1, respectively.). Samples were incubated with 5U RNase in reaction buffer for 15, 30 and 60 min. RNase activity was stopped and subsequently incubated with 50 μg of heparin sodium for 10 min.

ratio of apoptotic cells to the total number of cells, is presented in the lower right corner in each diagram. As shown in Fig. 7, naked siRNA provided no significant enhanced apoptotic effect in either cancer cell type. PLR/siRNA and PLR-DEX/siRNA exhibited an enhanced apoptotic effect n both cell lines, and PLR-DEX/siRNA induced the most significant effect, 40.22% and 50.47% in Hep-2 and FaDu cells, respectively. Additionally, this result can be correlated with efficient cellular uptake of and gene silencing by the PLR-DEX complex. Recently, it has been reported that cationic polymers, such as polyethyleneimine (PEI) and poly-L-lysine, could induce apoptotic effects in cancer cells (29). Since the apoptotic effect caused by scRNA and polymers was negligible (Supplementary Fig. 2), it can be concluded that the apoptotic effect of PLR-DEX with EGFR siRNA was caused primarily by the introduction of EGFR siRNA rather than the blank polymer complex.

In Vivo Anti-Tumor Efficacy

The anti-tumor effect of intratumoral injection of EGFR siRNA with or without developed nanocomplex was assessed in FaDu tumor xenografted mouse models. It has been reported that Lipofectamine 2000 with control siRNA might induce an off-target effect in *in vitro* transfection (30). Additionally, Lipofectamine 2000 has been reported to exhibit poor *in vivo* siRNA delivery efficiency (31); thus, it was not included in our *in vivo* studies. scRNA did not produce any significant *in vitro* gene silencing effect, and exhibited no serious systemic toxicity after intratumoral injection in our preliminary study (data not shown). Moreover, PLR-DEX/scRNA group was also not employed *in vivo* anti-tumor efficacy study because this group did not show a sufficient *in vitro* apoptotic effect. Tumor mass and body weight were measured for 20 days, and microscopic pictures of dissected tumors were taken after 20 days and immunohistochemical staining for the detection of EGFR expression and H&E staining were performed (Fig. 8). As shown in Fig. 8a, the tumor growth inhibition was ranked as PLR-DEX/siRNA > PLR/siRNA > naked siRNA > control after 20 days ($P< 0.05$). The relative tumor volume of PLR-DEX/siRNA, PLR/siRNA, and naked siRNA groups to that of the control group after 20 days were reduced to 8.72, 17.00, and 37.13%, respectively. Moreover, significant tumor growth inhibitory effect of developed nanocomplex system was observed on day 12 to 20 ($P<0.05$). This result coincided with the results of the cellular uptake study (Fig. 5) and EGFR gene silencing study (Fig. 6). Tumor size can also be observed in representative images (Fig. 8c) and dissected FaDu tumors after sacrifice (Fig. 8d). The mean values of body weight in all groups were maintained, with no sudden change (Fig. 8b). This indicated that developed nanocomplex exhibited no serious toxicity in the animal model and its use may be feasible for clinical application. The inhibitory effects of EGFR expressed on tumors were evaluated by immunohistochemical staining in the control and PLR-DEX/siRNA groups. As shown in Fig. 8e (upper panel), the dark brown color of DAB staining in the control group indicated EGFR expression in tumor, whereas an EGFR suppression effect was observed in the PLR-DEX/siRNA treatment group. The results of the H&E staining, lower panel in Fig. 8e, indicated that an apoptotic event occurred in the PLR-DEX/siRNA treatment group. In another report (32), EGFR siRNA delivery with tumor-targeted self-assembled nanoparticles produced EGFR silencing in tumor and NCI-H460 xenograft tumor growth inhibition. In that investigation, siRNA-loaded nanoparticles were injected by an intravenous route and siRNA was delivered by targeted nanoparticles. Due to the readily accessible nature of head and neck cancer, the EGFR siRNA formulation was injected intratumorally in this investigation and we achieved an excellent tumor growth inhibitory effect compared with previous results (32).

It is known that siRNA can be rapidly cleared from the body by nucleases degradation, renal excretion, and nonspecific uptake by the reticuloendothelial system

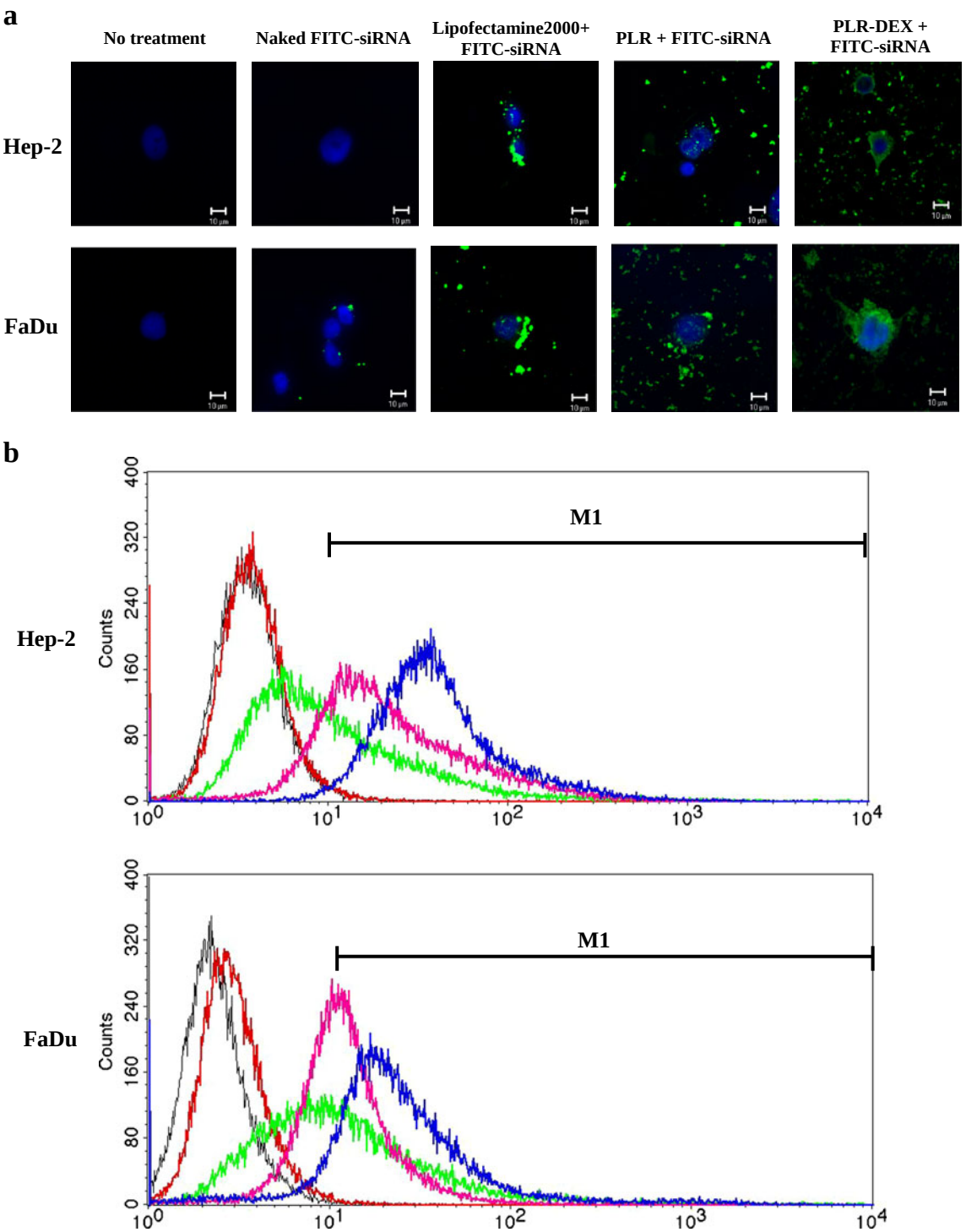


Fig. 5 *In vitro* cellular uptake study in Hep-2 and FaDu cells with FITC-labeled EGFR siRNA and/or polymer. (**a**) 100 nM of FITC-siRNA was incubated for 2 h and observed by CLSM. Merged images composed of FITC (*green color*) and DAPI (*blue color*) were shown. Groups were as follows: no treatment, naked FITC-siRNA, Lipofectamine 2000 + FITC-siRNA, PLR + FITC-siRNA (15:1, weight ratio), PLR-DEX + FITC-siRNA (10:1 and 15:1 for PLR:DEX and PLR-DEX:siRNA, weight ratio), (**b**) Cellular uptaken amounts of FITC-siRNA (100 nM) after 2 h of incubation were analyzed by flow cytometry. Various colors indicate several experimental groups (*black*: no treatment, *red*: naked FITC-siRNA, *green*: Lipofectamine 2000 + FITC-siRNA, *pink*: PLR + FITC-siRNA, *blue*: PLR-DEX + FITC-siRNA).

after its systemic administration (33). The mean diameter of the final nanocomplex formulation was adequate for the enhanced permeability and retention (EPR) effect in tumor tissue (34). Moreover, the *in vitro* stability

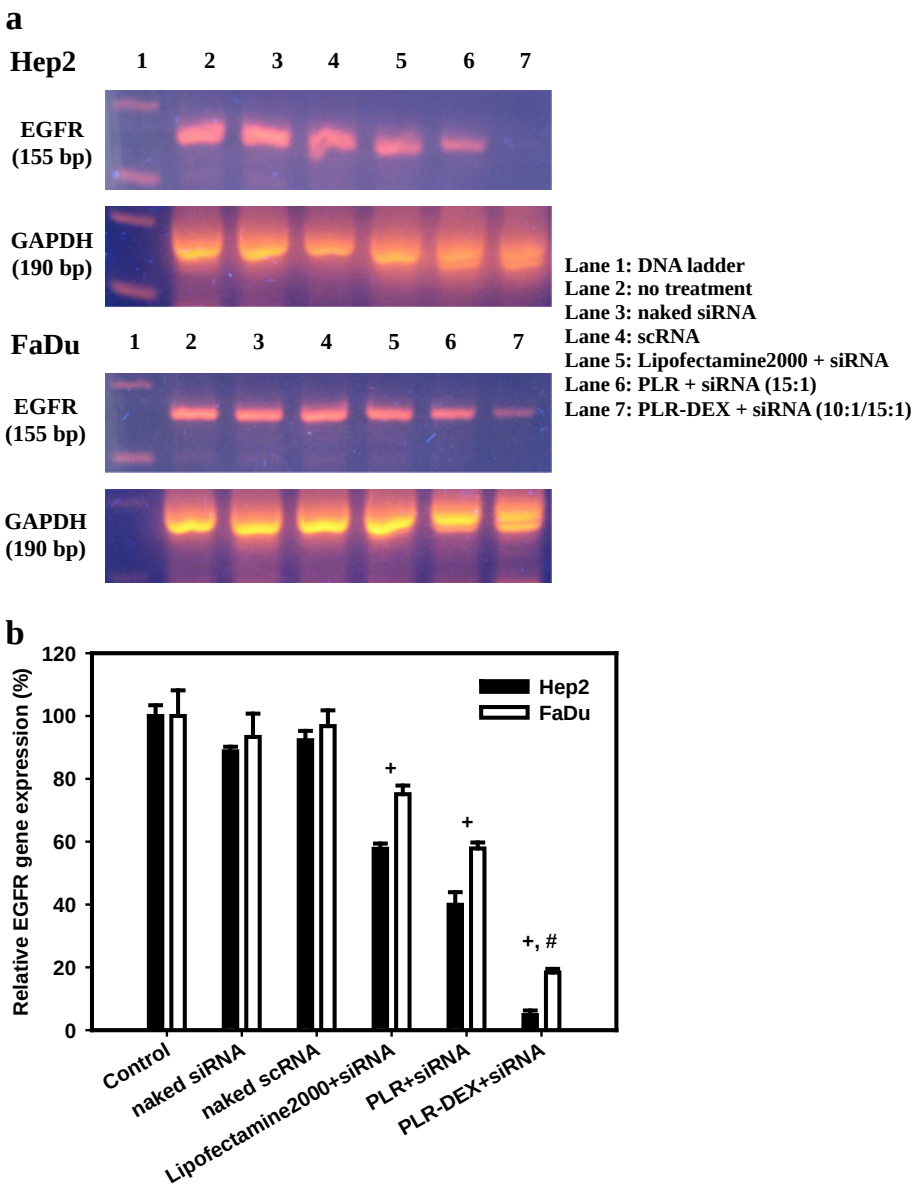


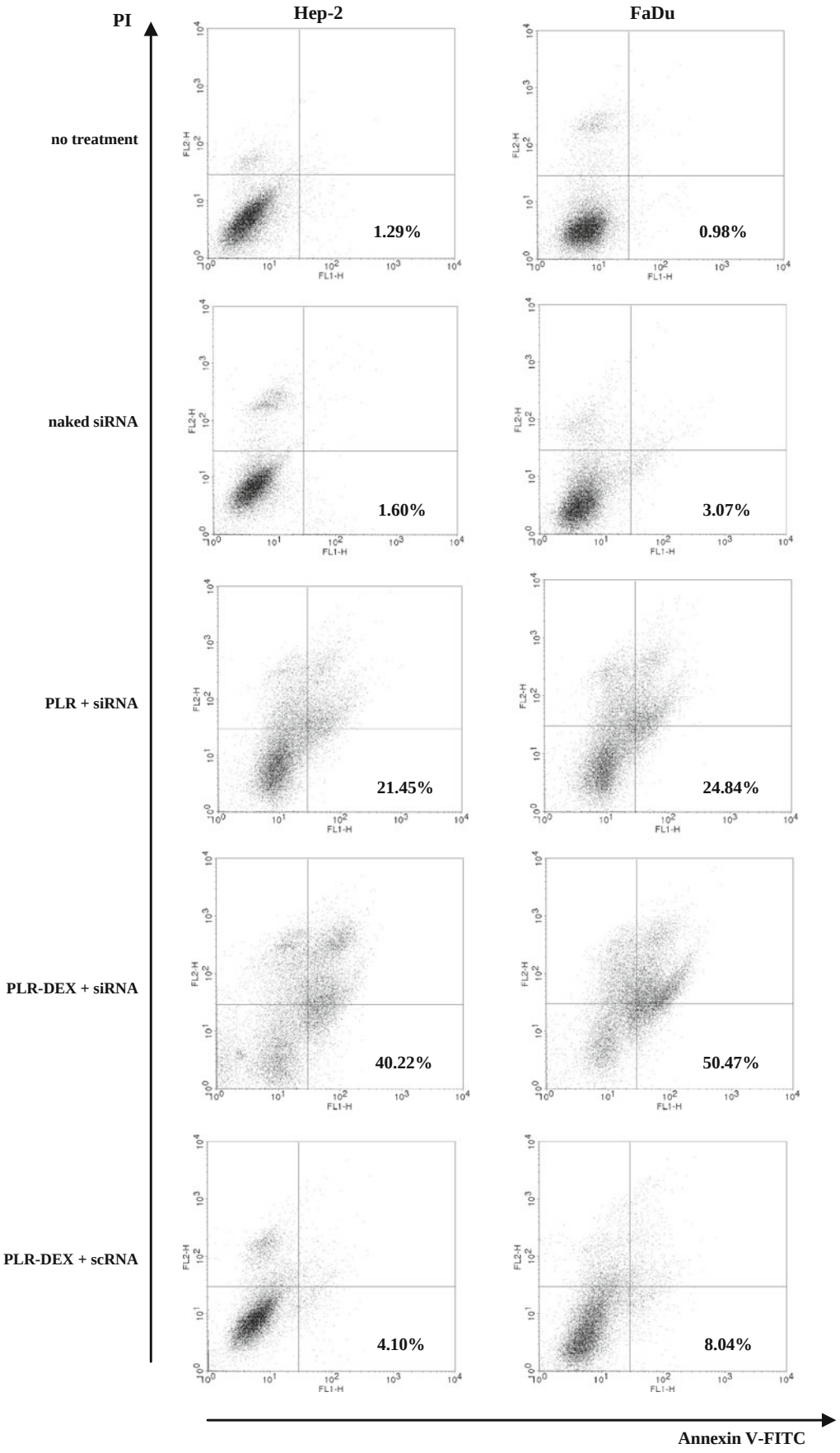
Fig. 6 *In vitro* gene silencing study in Hep-2 and FaDu cells. 100 nM of EGFR siRNA was contained in each formulation and it was incubated for 24 h. Gene silencing effect of EGFR siRNA in both cell lines was evaluated by RT-PCR. (**a**) PCR product was loaded onto a 2% agarose gel and observed by UV illuminator. (**b**) The ratio of EGFR mRNA to GAPDH mRNA in both cell lines was presented ($n=3$). No treatment group was considered as control group. $^{+}$ means $P<0.05$ compared to naked siRNA group in both cell lines.; $^{\#}$ means $P<0.05$ compared to PLR/siRNA group in both cell lines.

against RNase attack was maintained (Fig. 4). The *in vivo* stability of the nanocomplex in the body fluid is yet to be investigated and improved in order to be considered for systemic application. Nevertheless, the significance of the current study is in showing the silencing of EGFR by the new formulation, opening possibility for further development as an alternative therapeutic for head and neck cancer treatment.

CONCLUSIONS

EGFR siRNA-loaded nanocomplex, composed of PLR and DEX, was developed and evaluated in cell culture and in a tumor xenografted mouse model. The weight ratio between polymer and RNA was 15:1 (PLR:DEX=10:1) and the formation of a nano-sized complex was identified. The prepared PLR-DEX/siRNA provided highly efficient intracellular uptake and an EGFR gene silencing effect in Hep-2 and FaDu cells; its *in vitro* apoptotic effect was also demonstrated in both cancer cell lines. In a tumor xenografted mouse model, the anti-tumor efficacy of PLR-DEX/siRNA was identified following intratumoral injection. Therefore,

Fig. 7 Apoptotic effects of EGFR siRNA or negative siRNA (scRNA) with PLR and PLR-DEX complex. Hep-2 and FaDu cells were treated EGFR siRNA with or without polymer. X and Y axis mean the fluorescence of Annexin V-FITC and PI, respectively. The percentage of population presented in lower right side is the percentage of right panel in each group. Each value indicates mean value (n=3).


Hep-2
FaDu
PI
no treatment
naked siRNA
PLR + siRNA
PLR-DEX + siRNA
PLR-DEX + scRNA
1.29%
0.98%
1.60%
3.07%
21.45%
24.84%
40.22%
50.47%
4.10%
8.04%
FL2-H
FL1-H
Annexin V-FITC

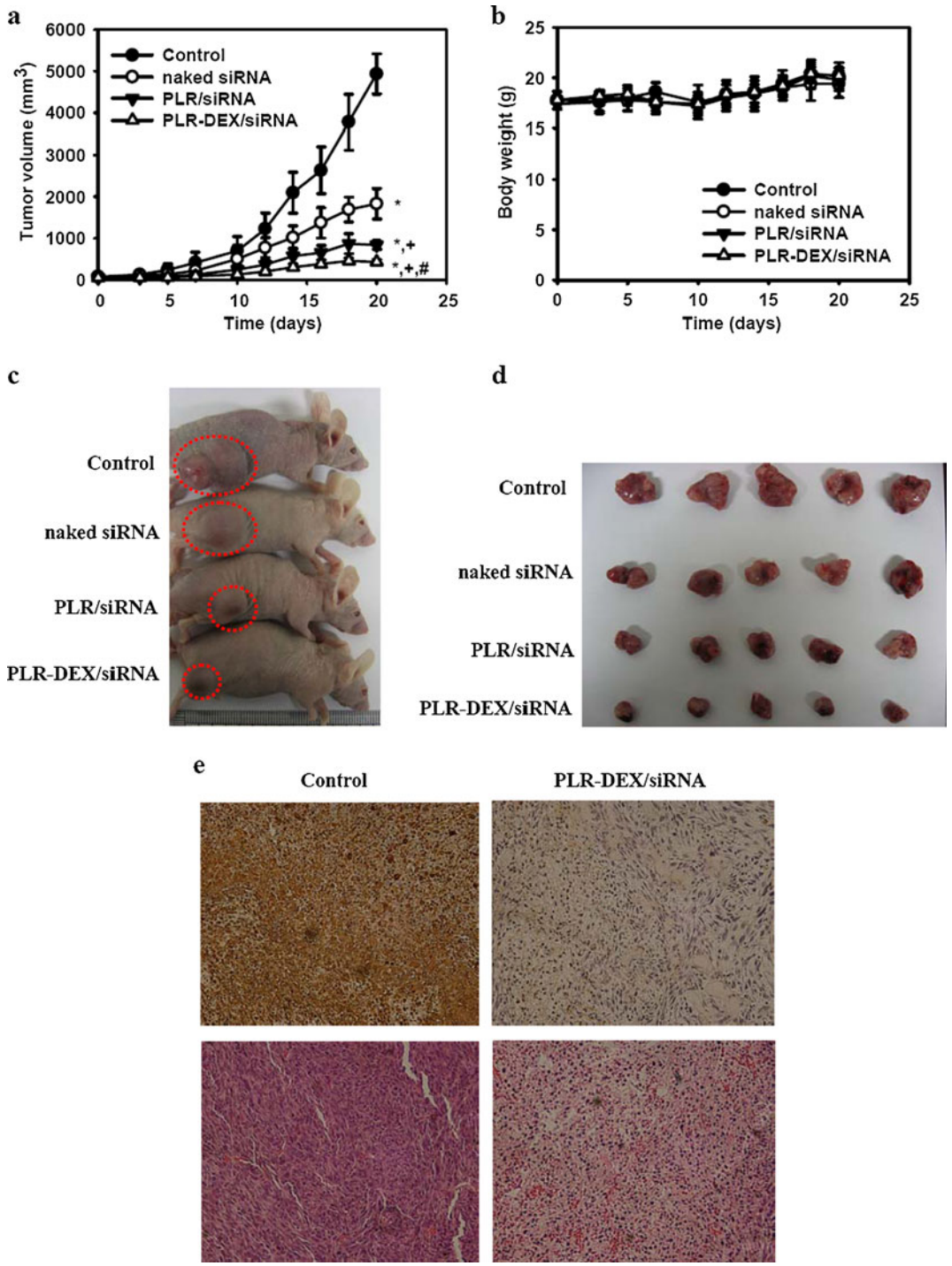


Fig. 8 Anti-tumor efficacy test of EGFR siRNA with or without polymer in a BALB/c mouse model where FaDu cells were inoculated for tumor growth. (**a**) Tumor volume (mm^3) profiles according to the time (days) were shown. EGFR siRNA was injected at 5, 7 and 10 days. Each point indicates mean ± S.D. ($n=5$). (**b**) Body weight (g) was also measured with tumor size measurement. Each point indicates mean ± S.D. ($n=5$). (**c**) Images of tumor xenografted mouse models after 20 days were exhibited. (**d**) Images of dissected tumor tissues after 20 days of treatment were shown. (**e**) The results of immunohistochemical staining for EGFR detection in tumor sections (*upper*) and hematoxylin and eosin (H&E) staining on tumor sections (*bottom*) were presented. Magnification = × 200. * $P<0.05$ compared to control group; $^{+}$ $P<0.05$ compared to naked siRNA group; $^{\#}$ $P<0.05$ compared to PLR/siRNA group after 20 days.

PLR and DEX-based nanocomplex containing EGFR siRNA was successfully developed and has shown that the new formulation was effective in EGFR gene silencing and tumor growth inhibition in head and neck cancer.

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