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Galactosylated Chitosan-g-PEI/DNA Complexes-loaded Poly(organophosphazene) Hydrogel as a Hepatocyte Targeting Gene Delivery System

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Hydrogels are widely used in drug delivery systems because they can control the release and thereby enhance the efficiency of locally delivered bioactive molecules such as therapeutic drugs, proteins, or genes. For gene delivery, localized release of plasmid DNA or polymer/DNA complexes can transfect cells and produce sustained protein production. We tested the galactosylated chitosan-graft-polyethylenimine (GC-g-PEI)/DNA complexes-loaded poly(organophosphazene) thermosensitive biodegradable hydrogel as a hepatocyte targeting gene delivery system. The poly(organophosphazene) hydrogel loaded with GC-g-PEI/DNA complexes showed low cytotoxicity and higher transfection efficiency than PEI/DNA complexes, as well as good hepatocyte specificity *in vitro* and *in vivo*. Our results indicate that poly(organophosphazene) hydrogels loaded with GC-g-PEI/DNA complexes may be a safe and efficient hepatocyte targeting gene delivery system.

Key words: Gene therapy, Galactosylated chitosan-g-polyethylenimine, Poly(organophosphazene) hydrogel, Hepatocyte targeting, Cytotoxicity, Transfection efficiency

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INTRODUCTION

Gene therapy provides enormous potential to treat human disease such as cancer and inherited genetic disorders (Yang et al., 2008). The success of gene therapy is largely dependent on the vector delivery system (Li et al., 2003). While most gene therapy protocols presently in clinical trials employ recombinant viral vectors, safety concerns such as immunogenicity and potential for mutagenesis of transfected cells have led to the pursuit of non-viral alternatives (Jiang et al., 2007a). Despite the advantages over viral vectors, including safety, lower immunogenicity, and the ability to transfer larger DNA molecules, most non-viral vectors suffer low gene transfection efficiency or significant toxicity (Park et al., 2006; Yang et al., 2008). These limitations have stimulated the search for new cationic polymer or gene delivery systems with high gene transfection efficiency and low cytotoxicity (Kushibiki and Tabata, 2004). Moreover, long-term gene expression decreases with cell division and intracellular degradation (Kushibiki and Tabata, 2004). Controlled-release gene delivery systems such as hydrogel systems or scaffold systems allow long-term gene expression (De Laporte and Shea, 2007; Klouda and Mikos, 2008; Lei and Segura, 2009; Scherer et al., 2002) because the controlled delivery of polymer/gene complexes enhances transfection efficiency by maintaining a microenvironment via sustained release (Shea et al., 1999).

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Thermosensitive hydrogels are a newer class of hydrogels that can exhibit a sol-gel transition at both room and elevated temperatures. These changes produce swelling or deswelling of the polymer and control release of encapsulated pDNA in response to external temperature changes (Holmes et al., 2000; Petka et al., 1998). Polyphosphazenes, thermosensitive hydrogels, have biomedical and pharmaceutical applications due to their excellent hydrolytic degradability and non-toxic degradability (Greish et al., 2005; Luten et al., 2003; Nukavarapu et al., 2008; Yang et al., 2008; Zhang et al., 2007).

In a previous study, we prepared galactosylated chitosan-graft-PEI (GC-g-PEI) as a gene carrier (Jiang et al., 2007b). GC-g-PEI showed low cell toxicity, high transfection efficiency, as well as high hepatocyte specificity. To improve control of gene delivery, here we loaded the GC-g-PEI/DNA complexes into polyphosphazene biodegradable hydrogels. We measured the release, cytotoxicity, and transfection efficiency of GC-g-PEI/DNA complexes *in vitro*, and hepatocyte specificity *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Chitosan (molecular weight, 100 kDa; deacetylation degree, 87.7%) was kindly supplied by Jakwang. Branched PEI 25K, galactose-bearing lactobionic acid (LA), potassium periodate, and DNA (sodium salt from calf thymus) were obtained from Sigma-Aldrich. Expression vectors, pGL3 (5.3 kb), containing a luciferase gene driven by an SV40 promoter, was purchased from Promega; and pEGFP-N2 (4.7 kb), encoding a green fluorescent protein driven by an immediate early promoter of CMV, was purchased from Clontech Laboratories. Branched PEI 1800Da was purchased from Wako. All other chemicals were reagent grade chemicals.

Synthesis and characteristics of poly(organophosphazene)

Poly(organophosphazene) was synthesized as previously reported (Lee, 2002; Park and Song, 2005). The viscosity measurement of aqueous polymer solution was performed on a Brookfiled RVDV-III + viscometer between 5.0 and 65.6°C according to a previously reported method (Kang et al., 2006).

Preparation of GC-g-PEI and release of GC-g-PEI/DNA complexes from poly(organophosphazene) hydrogel *in vitro*

The GC-g-PEI was synthesized as described previously (Jiang et al., 2007b). The release of GC-g-PEI/DNA complexes from poly(organophosphazene) hydrogel was examined using radioisotope-labeled DNA. Plasmid pGL3 DNA was radioisotope-labeled with ^{32}P dATP using a nick translation kit (Amersham Pharmacia Biotech) according to the manufacturer's protocols (Segura et al., 2003). The charge ratio (N/P=14) of the GC-g-PEI/DNA complexes was expressed as the ratio of moles of the amine groups of copolymer to moles of phosphates of DNA (Jiang et al., 2007b). All GC-g-PEI/DNA complexes were freshly prepared before use.

Poly(organophosphazene) was dissolved in PBS (0.01 M, pH 7.4, 10 wt-%) and then polymer/^{32}P-labeled DNA complexes were added at 4°C. The E-tubes containing the solution were incubated at 37°C, 100 rpm with PBS in a shaking incubator (SI-600R, JEIO TECH). An aliquot (0.5 mL) was withdrawn from the released medium and replaced by an equal volume of PBS at each sampling time. The released radioisotope-labeled DNA was determined using a Liquid Scintillation Counter (Packard), and then the total amount released was calculated from the established standard curve.

Cell lines, cell culture, and cell viability assays

HepG2 (human hepatoblastoma) and HeLa (human cervix epithelial carcinoma) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL). NCTC3749 (murine macrophage) cells were incubated in RPMI 1640 medium (Hyclone). All media was supplemented with 10% fetal bovine serum (FBS, HyClone), streptomycin at 100 μg/mL, and penicillin at 100 U/mL. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were split using trypsin/EDTA medium prior to reaching confluency.

In vitro cytotoxicity tests were performed using the Cell Titer 96® A_{queous} One Solution Cell Proliferation Assay (Promega) (Jiang et al., 2007a). Cells were seeded in 24-well plates at an initial density of 10×10^4 cells/well in 1 mL of growth medium and incubated for 24 h prior to addition of poly(organophosphazene) solutions. Growth media was replaced by fresh, serum-free media, containing various amounts of polymers. After an additional 24 h incubation, the media was replaced with growth media containing 20 μL of Cell Titer 96® A_{queous} One Solution Reagent. After 3 h incubation, absorbance at 570 nm was measured using an ELISA plate reader (GLR 1000, Genelabs Diagnostics). Cell viability (%) was calculated according to the following equation: cell viability (%) = $(OD_{570(sample)} / OD_{570(control)}) \times 100$, where $OD_{570(sample)}$ represents a measurement from a well treated with polymer and $OD_{570(control)}$ from a well treated with PBS buffer only.

Transfection study

Cells were seeded in 24-well plates at an initial density of 10×10^4 cells/well in 1 mL of growth medium and incubated for 24 h before transfection. From the release assay, the samples collected were adjusted and same amount of DNA (1 μg) was used for each transfection. Gene expression studies were evaluated as previously reported (Jiang et al., 2007a).

In vivo imaging study

Radio-labeling with ^{99m}Tc and imaging studies were performed as previously reported (Jiang et al., 2007b, 2008). *In vivo* imaging studies were performed in accordance with guidelines set by the Chonbuk National University Medical School Committee. Anesthesia was maintained during the imaging procedure. Six-week-old female BALB/c mice were injected with ^{99m}Tc-PEI/DNA or ^{99m}Tc-GC-g-PEI/DNA complexes (1 mCi/mouse) by i.p. administration (n=3). Static images were obtained at 5 min, 1, 3, and 7 days after i.p. injection using a gamma camera (Vertex, ADAC).

RESULTS AND DISCUSSION

Characterization of poly(organophosphazene)

Fig. 1. shows the gelation behavior of poly(organophosphazene) in PBS (pH 7.4, 37°C) by measuring viscosity as a function of temperature. The poly(organophosphazene) showed a reversible four-phase transition with temperature (Kang et al., 2006; Lee et al., 2004).

Release of polymer/DNA complexes from poly (organophosphazene)

The release of the naked DNA, PEI/DNA complexes, and GC-g-PEI/DNA complexes from poly(organophosphazene) *in vitro* showed similar patterns as they are mostly controlled by the hydrophilic poly(organophosphazene) property, with sustained release until two weeks through a zero-order pattern. pDNA release from the gel is mainly regulated by diffusion (Kasper et al., 2005; Wieland et al., 2007). Plasmid released from the hydrogels had two distinct bands pertaining to both the supercoiled and relaxed forms, reflecting maintenance of structural integrity of the plasmid in the release medium and the hydrogel (data not shown).

Cell toxicity of poly(organophosphazene)

Poly(organophosphazene) has low cytotoxicity after 24 h at 1 mg/mL, showing 100% viability in HepG2, NCTC3749, and HeLa cell lines compared to control (Fig. 3). Higher levels showed some toxicity (70% cell viability at 10 mg/mL), and higher concentrations were too viscous to test.

Cell transfection

HepG2 cells are models of parenchymal cells in the liver, which have good cell-surface expression of ASGPR. GC-g-PEI showed higher transfection efficiency than PEI 25K, suggesting that the GC-g-PEI carrier system is more efficient than the PEI one (Fig. 4). Similar transfection efficiencies in 1, 3, and 7-day samples suggest that the plasmids in poly(organophosphazene) are protected. GC-g-PEI/DNA complexes showed higher transfection efficiency in HepG2 cells than CHI-g-PEI/DNA ones, indicating that the galactose ligands on GC-g-PEI played a significant role in ASGPR recognition and enhanced transfection efficiency in HepG2 cells (Fig. 5).

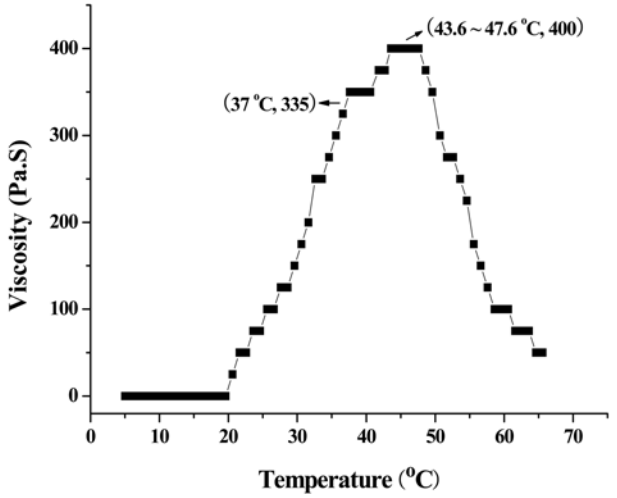

Fig. 1. Viscosity changes of poly(organophosphazene) hydrogel.

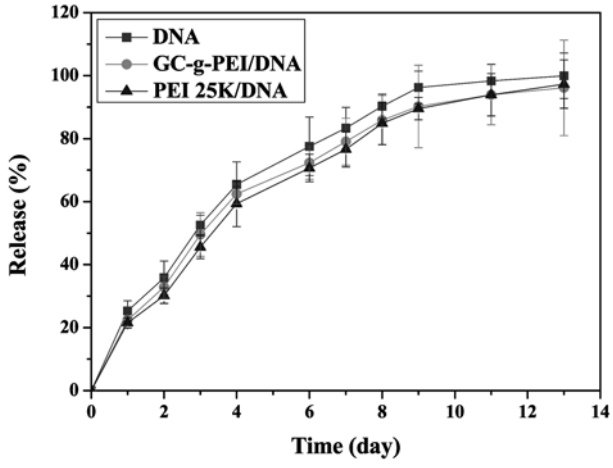

Fig. 2. Release profile of polymer/DNA complexes from poly(organophosphazene) hydrogel.

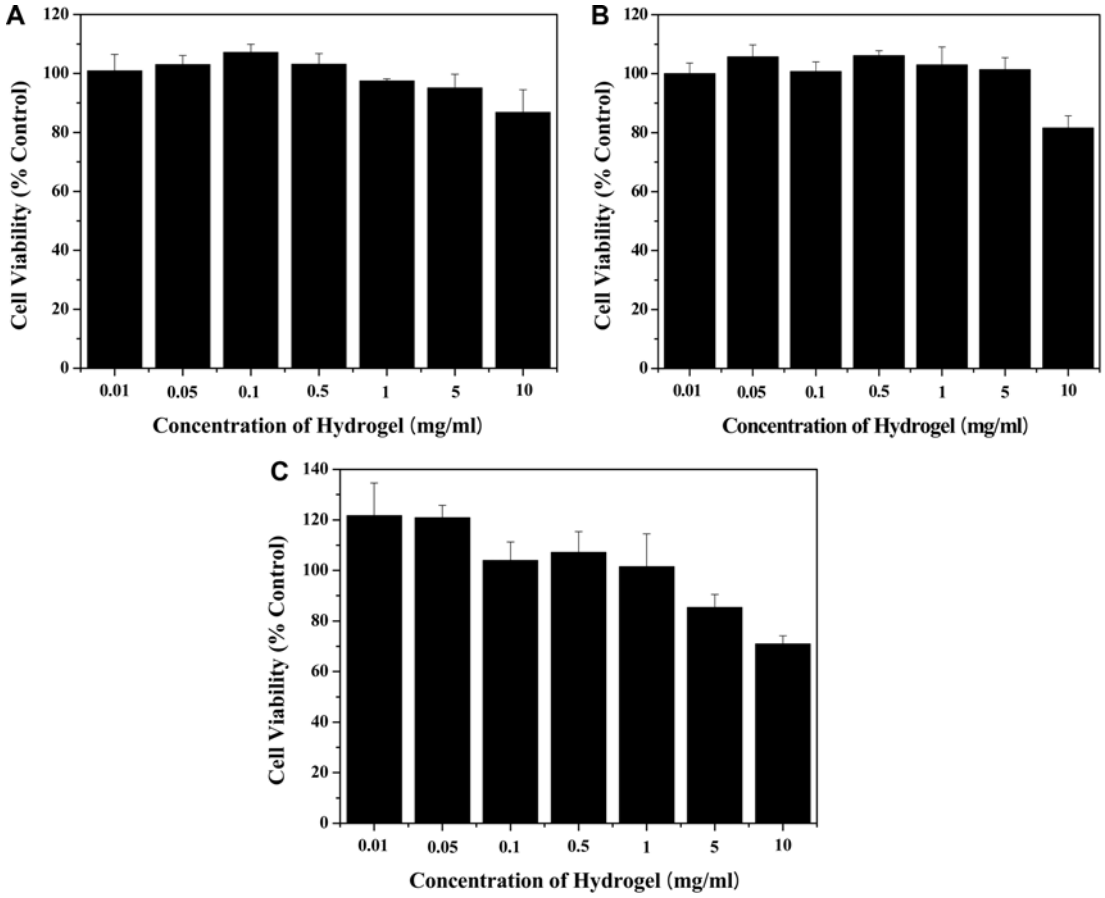

Fig. 3. Cytotoxicity of poly(organophosphazene) in different cell lines. (**A**) HepG2, (**B**) NCTC3749, and (**C**) HeLa (n=3, error bars represent standard deviation).

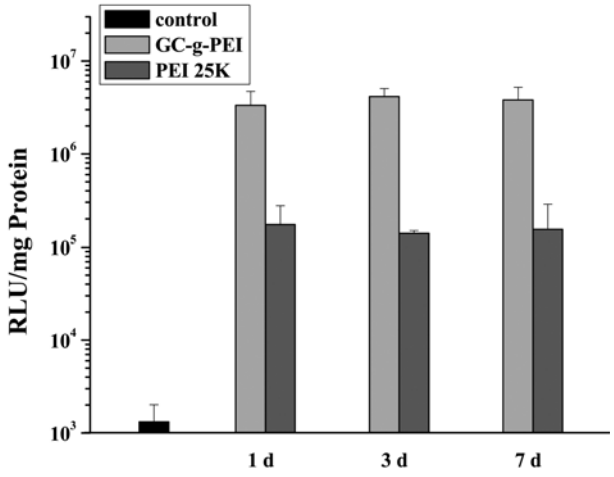

Fig. 4. Transfection efficiency of GC-g-PEI/DNA complexes in poly(organophosphazene) hydrogels in HepG2 cells (n=3, error bars represent standard deviation).

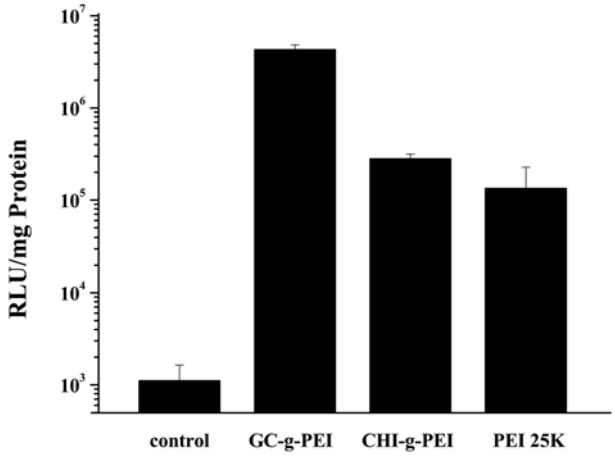

Fig. 5. Luciferase activity of GC-g-PEI/DNA and CHI-g-PEI/DNA complexes on HepG2 cells (n=3, error bars represent standard deviation).

In vivo distribution of ^{99m}Tc-labeled polymer/DNA complexes-loaded hydrogel

After i.p. injection to six-week-old female BALB/c mice, the ^{99m}Tc-labeled polymer/DNA complexes were slowly released from the poly(organophosphazene) hydrogels and accumulated in the liver. Also, more ^{99m}Tc-GC-g-PEI/DNA complexes accumulated in the liver over time than ^{99m}Tc-PEI/DNA, indicating better hepatocyte targeting and gene delivery.

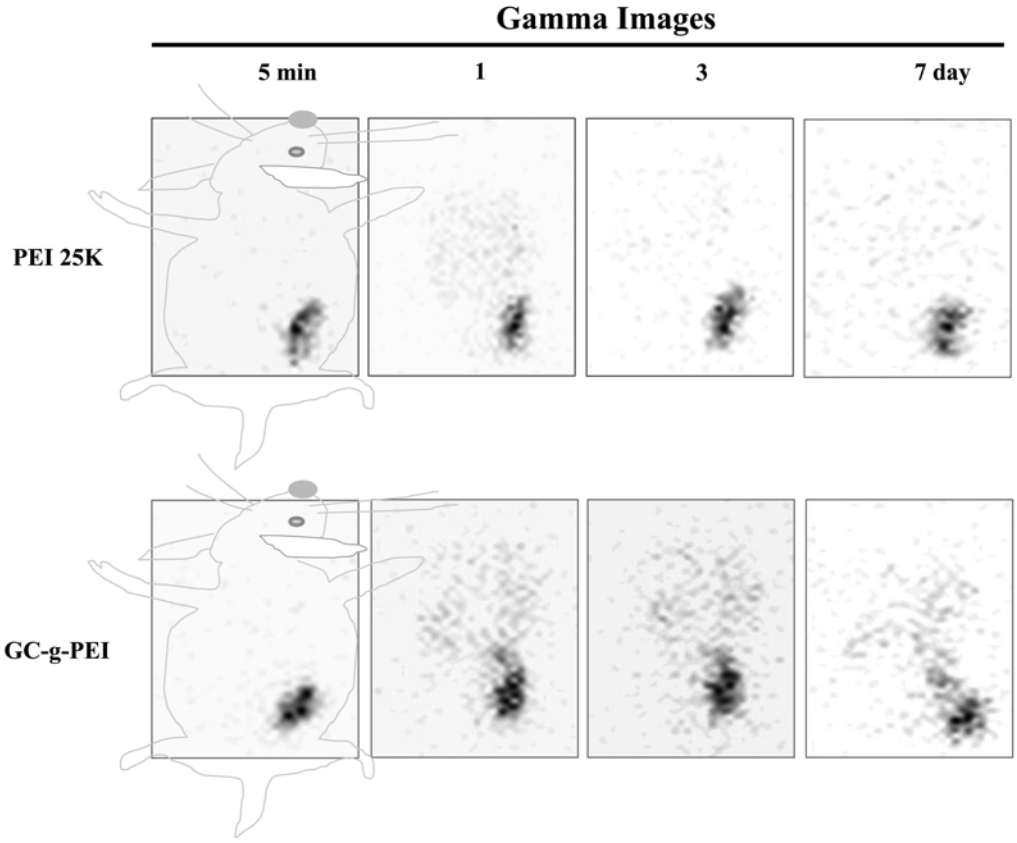

Fig. 6. Gamma images of mice treated with i.p. administration of ^{99m}Tc-PEI/DNA complexes and ^{99m}Tc-GC-g-PEI/DNA complexes in poly(organophosphazene) hydrogels.

In summary, thermosensitive, biodegradable poly (organophosphazene) hydrogel provides a safe and efficient liver delivery system for sustained delivery of pDNA and GC-g-PEI/DNA complexes.

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