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Galactosylated poly(ethylene glycol)-chitosan-graft-polyethylenimine as a gene carrier for hepatocyte-targeting

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ABSTRACT

Chitosan and chitosan derivatives have been proposed as alternative and biocompatible cationic polymers for non-viral gene delivery. However, the low transfection efficiency and low specificity of chitosan is an aspect of this approach that must be addressed prior to any clinical applications. In the present study a chitosan derivative, galactosylated poly(ethylene glycol)-chitosan-graft-polyethylenimine (Gal-PEG-CHI-g-PEI), was investigated as a potential hepatocyte-targeting gene carrier. The composition of Gal-PEG-CHI-g-PEI was characterized using ^{1}H nuclear magnetic resonance (^{1}H NMR), and the particle size and zeta potential of Gal-PEG-CHI-g-PEI/DNA complexes were measured using dynamic light scattering (DLS). The Gal-PEG-CHI-g-PEI exhibited lower cytotoxicity compared to PEI 25K as a control. Likewise, Gal-PEG-CHI-g-PEI/DNA complexes showed good hepatocyte specificity. Furthermore, Gal-PEG-CHI-g-PEI/DNA complexes transfected liver cells more effectively than PEI 25K *in vivo* after intravenous (i.v.) administration. Together, these results suggest that Gal-PEG-CHI-g-PEI, which has improved transfection efficiency and hepatocyte specificity both *in vitro* and *in vivo*, may be useful for gene therapy.

1. Introduction

The success of gene therapy is limited mainly by the lack of suitable vectors able to deliver DNA to targeted cells. For example, while viral gene delivery vectors have high transfection efficiencies over a wide range of cell targets, they have major limitations, including virally-induced inflammatory responses and oncogenic effects. To circumvent these obstacles, gene delivery research has focused on the development of non-viral gene delivery vectors [1]. Of the presently-studied non-viral vectors, chitosan and chitosan derivatives have been proposed as alternative and biocompatible cationic polymers that are suitable for non-viral gene delivery [2]. However, this system is significantly limited by the low transfection efficiency and low cell specificity of chitosans [3,4]. To address these limitations, several ligands such as transferrin- [5,6], folate- [7,8], mannose- [9] and galactose- [10,11] conjugated chitosans have been designed and evaluated for receptor-mediated endocytotic gene delivery. Of these ligand-conjugated chitosans, galactosylated chitosans have been reported as hepatocyte-targeting gene carriers due to specific ligand–receptor interactions between galactose-moieties and asialoglycoprotein receptors (ASGPRs). The ASGPRs are present at a high density only on hepatocytes, and are retained on several human hepatoma cell lines [12–14]. Another type of non-viral vector, polyethylenimine (PEI), has been shown to condense plasmids into colloidal particles that can effectively transfect a variety of cells, both *in vitro* and *in vivo*, due to its buffering capacity [15]; however, many studies have raised concerns regarding the toxicity of conventional PEI, which is dependent on its molecular weight; lower molecular weight PEI has a lower cytotoxicity [16,17].

In a previous study, we prepared chitosan-graft-PEI (CHI-g-PEI) as a gene carrier [18]. The CHI-g-PEI exhibited low cell toxicity and high transfection efficiency; however, this system also has limited cell specificity. Therefore, in this study, we prepared Gal-PEG-chitosan-graft-low molecular weight PEI (Gal-PEG-CHI-g-PEI) to achieve better hepatocyte specificity. In general, PEG facilitates the formation of polyplexes with improved solubility, lower surface charge, diminished aggregation, lower cytotoxicity, and possibly decreased opsonization

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with serum proteins in the bloodstream [19]. The physicochemical properties of Gal-PEG-CHI-g-PEI/DNA complexes were analyzed and their cytotoxicity and hepatocyte specificity were also characterized. Transfection efficiency was investigated both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Chitosan (molecular weight, 100 kDa; deacetylation degree, 87.7%) was kindly supplied by Jakwang (Ansung, Korea). A hetero-bifunctional poly(ethylene glycol) derivative (NH_2-PEG-COOH, MW 5000) was purchased from NOF Cooperation (Tokyo, Japan). Lactobionic acid (LA) and branched PEI 25K were obtained from Sigma-Aldrich (St. Louis, MO, USA). Branched PEI 1800 Da was purchased from Wako (Osaka, Japan). All other chemicals were of reagent grade.

2.2. Preparation of Gal-PEG-CHI-g-PEI

The Gal-PEG-CHI-g-PEI copolymer was synthesized in two steps. In the first step, galactosylated PEG was synthesized by an amide formation reaction between activated carboxyl groups of galactose-bearing LA and amine groups of bi-functional PEG. Briefly, LA (5 mmol) dissolved in 50 ml of MES buffer solution (0.1 M, pH 6.5) was activated with a mixture of NHS (20 mmol) and EDC (20 mmol). After activating the carboxyl groups for 15 min, 0.5 mmol of PEG was added. The reaction was run at 12 h at 4 °C followed by an additional 12 h at room temperature. The resulting product was purified by dialysis (3500, MWCO) against distilled water for 3 days, followed by lyophilization. CHI-g-PEI was synthesized as previously described [18].

In the second step, Gal-PEG-CHI-g-PEI was synthesized by an amide formation reaction between activated carboxyl groups of Gal-PEG and amine groups of CHI-g-PEI. Gal-PEG (0.1 mmol) dissolved in 10 ml of MES buffer solution (0.1 M, pH 6.5) was activated with a mixture of NHS (1 mmol) and EDC (1 mmol). After activating the carboxyl groups for 15 min, 0.001 mmol of CHI-g-PEI was added. The reaction was allowed to run at 12 h at 4 °C followed by an additional 12 h at room temperature. The resulting product was purified using a dialysis tube (12,000, MWCO) against distilled water for 3 days. After dialysis, the copolymer was lyophilized. The reaction scheme is shown in Fig. 1.

2.3. Characterization of copolymer

The composition of the prepared Gal-PEG-CHI-g-PEI copolymer was estimated by measuring ^{1}H nuclear magnetic resonance (^{1}H NMR) (AVANCE™ 600 FT-NMR, Bruker, Germany). The molecular weight of the Gal-PEG-CHI-g-PEI copolymer was measured using a GPC operating at a wavelength of a 690 nm (Dawn Eos, Wyatt, USA).

2.4. Preparation and characterization of Gal-PEG-CHI-g-PEI/DNA complexes

All Gal-PEG-CHI-g-PEI/DNA complexes were freshly prepared before use. Gal-PEG-CHI-g-PEI/DNA complexes were prepared by combining equal volumes of a DNA solution and copolymer solution, gently vortexing the solution, and incubating it at room temperature for 30 min.

Characterization of Gal-PEG-CHI-g-PEI/DNA complexes was performed using methods previously reported [18]. Briefly, the DNA condensation ability of the copolymer was confirmed by electrophoresis. DNA retardation was observed by irradiation with UV light and analyzed with Cam2com software. The morphologies of the Gal-PEG-CHI-g-PEI/DNA complexes were observed using energy-filtering transmission electron microscopy (EF-TEM) (LIBRA 120, Carl Zeiss, Germany). The sizes and surface charges of Gal-PEG-CHI-g-PEI/DNA complexes were measured at room temperature using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka Electronics, Osaka, Japan) at scattering angles of 90 and 20°, respectively.

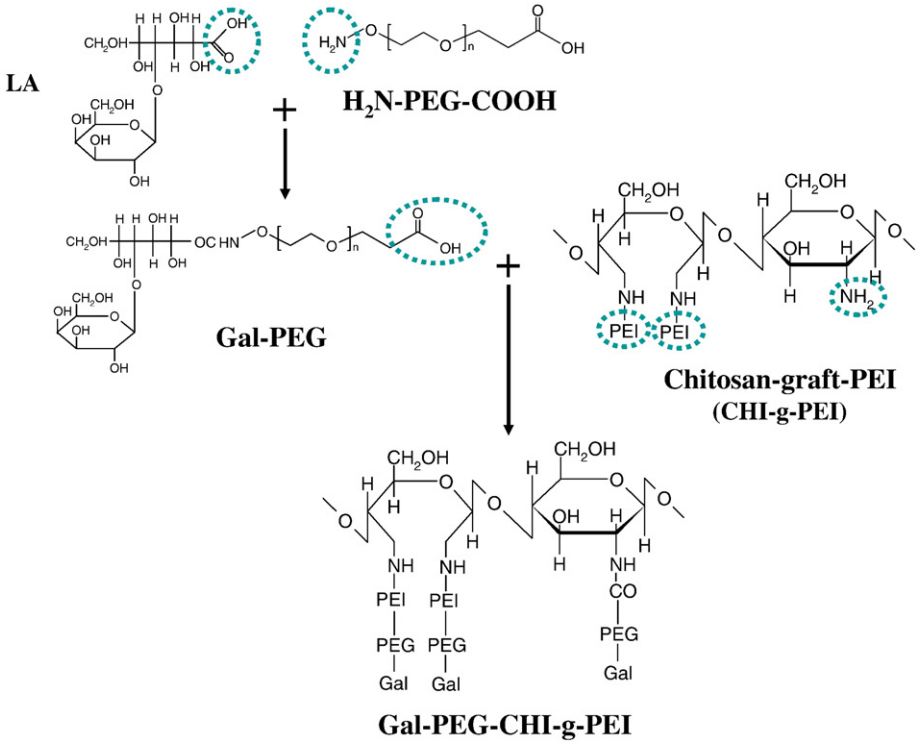

Fig. 1. Proposed reaction scheme for synthesis of Gal-PEG-CHI-g-PEI.