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"Click" chemistry mediated construction of cationic curdlan nanocarriers for efficient gene delivery

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ABSTRACT

A cationic group has been quantitatively and selectively introduced into C6 position of each glucose units of Curdlan by "Click Chemistry" successfully. The resulting cationic Curdlan-Imidazole-lysine polymers (Cur-6-100Lys) exhibit excellent water solubility. Structure of the Cur-6-100Lys complexes was verified by FTIR and NMR spectroscopic measurements, and analysis of Cur-6-100Lys by GPC, DLS and SEM revealed that they have stoichiometric, nanosized spheroidal structures. Cytotoxicity measurement, electrophoretic mobility shift assay and EGFP-pDNA transfection have been carried out respectively. The results clearly show that Cur-6-100Lys nanocarriers have bound to dsDNA promptly, are less cytotoxic to both 7901 cells and HeLa cells, and are readily able to transport EGFP-pDNA into HepG2 cells. Our studies indicated that Cur-6-100Lys can potentially be used as a versatile nano platform for efficient gene delivery in living cells.

1. Introduction

Gene-based therapy is expounded as the treatment of human diseases by the transfer of genetic material into specific cells of patients to modulate gene expression (Mulligan, 1993; Pack, Hoffman, Pun, & Stayton, 2005). The current achievements demonstrate that such gene regulation can be accomplished by exogenous nucleic acids such as DNA, mRNA, small interfering RNA (siRNA), microRNA (miRNA) or antisense oligonucleotides (Yin et al., 2014). During the past 20 years, given the rapid advances in molecular biology, and the great achievement of Human Genome Project, a variety of diseases (such as haemophilia (Walsh, 2003), muscular dystrophy (van Deutekom & van Ommen, 2003), cancer, angiocardiopathy) have been heavily investigated to treat or prevent them by gene-based therapy. Nevertheless, applying gene therapy in clinical trials in large-scale has been limited owing to considerable technical barriers. Taken their large size, the negative charge and high sensitivity to biological environment, delivering therapeutic genes are typically mediated by carriers or vectors. A fundamental technical limiting obstacle to clinical application of gene therapy is the lack of safe and efficient gene delivery carriers.

To date, besides viral vectors, a wide range of materials (from inorganic to organic) and their functionalized derivatives provide alternative platforms for gene delivery in vitro and in vivo (Akinc, Thomas, Klibanov, & Langer, 2005; Copolovici, Langel, Eriste, & Langel, 2014; Tiera, Shi, Winnik, & Fernandes, 2011), and all sorts of synthetic or semi-synthetic material-based nanoparticles (NPs) have been created to overcome some of the obstacles in terms of gene stability in the body and efficiency of organ targeting and tissue penetration (Jayaraman et al., 2012; Jiang et al., 2013; Jokerst, Lobovkina, Zare, & Gambhir, 2011). Polysaccharides comprise an implausibly diverse family of natural polymers. There are a lot of potential reactive groups existing on polysaccharide structure units that is endowed with special properties ideal for use in research and development of stealth materials. (Liu & Edgar, 2015) Additionally, natural polysaccharides are basically biodegradable and have excellent biocompatibility. In particular, cell uptake efficiency of the resultant polysaccharide/polynucleotide complexes was remarkably enhanced when functional groups exist. That unique property of polysaccharide has made it possible to be utilized as potential carriers for various functional polynucleotides, resulting in some significant research progresses. Used as siRNA delivery vehicle, cyclodextrin nanoparticles (Davis et al., 2010) have been investigated and applied on human for RNAi therapy. Anisamide-targeted cyclodextrin nano formulation for siRNA delivery to prostate cancer cells expressing the sigma-1 receptor was systematically studied. (Godinho, Ogier, Darcy, O'Driscoll, & Cryan, 2013)

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Chitosan nanoparticles loaded with VEGF-siRNA have shown their possible advantages for gene silencing in vivo (Yang et al., 2011). Polyethylenimine (PEI)-pullulan functionalized with folic acid has been explored as non-viral carrier and indicated excellently targeted toward HeLa cell (Wang, Dou, & Bao, 2014). Curdlan belongs to the family of (1 → 3)-β-glucans, which is a kind of linear structure composed of D-glucose monomers connected by glycosidic linkages. Because Curdlan is nontoxic and has high biocompatibility (physicochemical and biological properties) to mammals, Curdlan and their derivatives have been developed on potential biomedical and pharmaceutical applications including drug-impregnated gels (Kanke, Tanabe, Katayama, Koda, & Yoshitomi, 1995), immune modulation (Kurita, Matsumura, Takahara, Hatta, & Shimojoh, 2011) and antiviral materials (Jagodzinski et al., 1994; Yoshida et al., 1995).

Recently, we reported successfully preparation of a potential siRNA nanocarrier by amino-functionalized Curdlan (6-deoxy-6-amino Curdlan, 6AC-100) based nanoparticle, which expeditiously delivered siRNA to multiple cell lines through electrostatic interaction, and significantly knocked down endogenous gene level (Han et al., 2015). Afterwards, Galactose-functionalized 6-amino-6-deoxy-Curdlan (6AC-100Lac) and PEGylation of 6-amino-6-deoxy-curdlan (6AC-100PEG) with less cytotoxicity than the parent molecule 6AC-100, have been successively synthesized by chemical modification of 6AC-100. The results of siRNA delivery in vitro manifested that 6AC-100Lac with higher substitute degree preferred to transport siRNA into HepG2 cells (expressing ASGPR receptor) through receptor-mediated internalization, and considerably knocked down endogenous gene (Wu, Cai, Han, & Baigude, 2015). 6AC-100PEG nanoparticles preferably carried siapoB into liver and down-regulated apoB mRNA and apoB 100 protein level significantly according to results of systematical delivery in mouse (Altangerel et al., 2016). Despite efforts, those direct modifications of 6AC-100 were unable to figure out strategies for conflicts between functionalized modification and gene loaded capacity. Therefore, it is vitally necessary to use a novel approach to construct curdlan functionalized vehicles with higher gene loading capacity.

Herein, we report an alternative convenient way to prepare curdlan grafted cationic polymers using “click chemistry”: Cu(I)-catalyzed chemo-selective [3 + 2]- cycloadditions between 6-azido-6-deoxy-curdlan and modified lysine with terminated-alkyne (Ikeda et al., 2007; Kolb, Finn, & Sharpless, 2001). In order to evaluate the potential application of Cur-6-100Lys nanospheres as gene vehicles, the cytotoxicity and the gene capacity are studies, and pDNA is chosen as a model gene to investigate gene delivering behaviors in vitro. The general diagram is shown in Scheme 1.

2. Materials and methods

2.1. Chemicals and general methods

Curdlan was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were purchase from Aladdin (Shanghai, China). Dimethylformamide (DMF) was distilled after drying. Dimethylsulfoxide (DMSO) was dried over 4 Å molecular sieve. The Fourier transform infrared (FTIR) spectrum of Cur-6-100Lys was recorded using NEXUS-670 FTIR instrument at room temperature using KBr pallet. ^{13}C NMR was recorded on a Brucker 500 NMR spectrometer. Chemical shifts (δ = 0 ppm) were referred to TMS with the residual proton of the deuterated solvent. Gel permeation chromatography (GPC) was performed on a Waters HPLC instrument equipped with Ultrahydrogel^{TM}500 ($7.8 \times 300\,mm^2$) and Ultrahydrogel^{TM}250 ($7.8 \times 300\,mm^2$) columns (Waters) using acetate buffer (pH 4.5) as eluent.

2.2. Synthesis of Cur-6-100Lys conjugates

Cur-6-100Lys conjugate was synthesized from curdlan and lysine in three steps: (i) Treated curdlan with LiBr Ph_3P and NBS sequentially, then reacted with NaN_3, which converted all C6-OH of curdlan into C6-N_3 groups. (ii) Carried out reaction between Propargylamine and Boc-lysine-OSu, after Boc group deprotected, to obtain PA-Lys with terminal alkynyl. (iii) Alkynyl −PA-Lys conjugated with N_3-curdlan to yield Cur-6-100Lys in which lysine was linked to curdlan via an 1, 2, 3- triazole ring.

2.2.1. Preparation of 6-azido-6-deoxy-curdlan

Dry curdlan (0.5 g) was dissolved in 20 mL dimethylformamide (DMF) containing LiBr by stirring at 80 °C for 3 h. After the mixture was cooled to room temperature, Ph_3P solution (2.99 g of Ph_3P in 2 mL of dry DMF) was added drop wise via a liquid addition funnel. Then NBS solution (2.19 g of *N*-Bromosuccinimide in 2 mL of dry DMF) was added into the mixture in a similar fashion mentioned above. After the reaction mixture was stirred for 12 h at 70 °C under nitrogen atmosphere, 1.0 g of NaN_3 was added carefully, and the reaction mixture was stirred at 70 °C for 24 h under the nitrogen atmosphere. When the reaction was completed, the product was separated by precipitation using methanol, followed by washing with water for 3 times. The product was further purified by reprecipitation from a mixed solution of methanol, alcohol, and water. A brown crystal (0.45 g) was obtained in 80% yield.

2.2.2. Preparation of PA-lysine

(i) Preparation of Di-Boc-lysine: 4.55 g of L-lysine hydrochloride was completely dissolved in 40 mL of 1, 4 dioxane and pure water (10 mL) in the reaction bottle, then added 70 mL of NaOH solution (1 M), the above mixture was stirred for 30 min at 0 °C.12.5 mL of Di-*tert*-butyl-dicarbonate dropped in and agitated till the mixture clarification, and was stirred for 12 h at room temperature. Finally, the reaction mixture was distilled in vacuum, and diluted with DCM (40 mL), washed with 5% of sodium citrate solution, distilled water and saturated sodium chloride solution in sequence. The as-obtained sample was dried, filtered and concentrated in a vacuum, and was denoted as Di-Boc-lysine.

(ii) Activation of carboxyl of Di-Boc-lysine: 8.45 g of Di-Boc-lysine was dissolved in 40 mL of anhydrous dimethylformamide, followed by addition of NHS (3.39 g), EDC (5.61 g) and DIAP (4.84 mL). The mixture was stirred for 12 h at room temperature under a nitrogen atmosphere, and the reaction was monitored by thin-layer chromatography (TCL) using dichloromethane and methylene as developing solvent. When the reaction completed, the solvent was removed in vacuo, and pale yellow oily residues were dissolved with DCM. The obtained mixture was washed with 5% of sodium citrate solution, distilled water and saturated sodium chloride solution in sequence. After dried over anhydrous sodium sulfate for 1 h, the mixture was purified by column chromatography over silica gel (DCM/MeOH: 20/1) to separate Di-Boc-lys-osu solution, then concentrated and lyophilized.

(iii) Synthesis of Di-Boc-lysine-PA: Propargylamine (200 mg), di-Boc-lysine-Osu (1.93 g), and DIAP (720 μL) were dissolved in DMF under a nitrogen atmosphere, and stirred for 2 h at room temperature. The reaction was monitored by TCL using mixtures of dichloromethane and methanol as an eluent. After completion of the reaction, the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane and washed with aqueous citric acid solution, water, and saturated NaCl solution. The solution was dried over anhydrous Na_2SO_4. After work-up, the product was purified by column chromatography over silica gel using a mixture