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A pH-sensitive gene delivery system based on folic acid-PEG-chitosan – PAMAM-plasmid DNA complexes for cancer cell targeting

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ABSTRACT

In this study, pH-sensitive biomaterials coated polymer/DNA nanocomplexes containing a high mobility group box 1 (HMGB1) were developed as an efficient non-viral gene delivery system. HMGB1 is a family of endogenous molecules that contains nuclear locating sequences (NSL). Polyethylene glycol tethered carboxylated chitosan modified with folic acid (FA-PEG-CCTS) was synthesized and its buffering capacity was determined by acid–base titration. A pH-sensitive core–shell system FA-PEG-CCTS/PAMAM/HMGB1/pDNA nanocomplexes (FPCPHDs), was prepared and characterized. Electrophoresis showed that FPCPHDs were resistant to heparin replacement and DNase I digestion. FPCPHDs exhibited only minor toxic effects on HepG2 and KB cells. The results of both luciferase activity assay and RFP fluorescence intensity analysis showed that FPCPHDs enhanced gene transfection and expression in KB cells. Moreover, gene transfection and expression in KB cells were inhibited by free folic acid. Intracellular trafficking of FPCPHDs in KB cells showed that FPCPHDs could rapidly escape from endo-lysosomes and become exclusively located in the nucleus at 3 h post transfection. In addition, FPCPHDs exhibited increased red fluorescence protein (RFP) expression at the tumor site of S180 xenograft nude mice. All results suggest that FPCPHDs is an efficient approach to improve the transfection and expression efficiency in most FR-positive cancer cells.

1. Introduction

Non-viral gene delivery systems have several advantages such as low immunogenicity, ability to deliver large-size genetic materials and the potential for modification of the surface physicochemical properties associated with biological interaction. However, the major limitations of non-viral gene transfer in vivo reside in the obstacles in biological systems during the period of physiologic circulation and targeting the designated tissues or cells, or even subcellular organelles [1,2]. These barriers include avoiding hepatic uptake and interaction with plasma components, binding to the cell surface, crossing the plasma membrane, avoiding endosomal degradation, and overcoming the nuclear envelope [3].

Unlike siRNA and shRNA, plasmid DNA (pDNA) needs to reach the nucleus where transcription takes place. So, nuclear location and access is certainly a main obstacle for successful delivery both in vitro and in vivo since the arrival of a plasmid DNA at the nucleus by a passive process is a rare event [1,4]. Peptides and proteins with a nuclear locating signal (NLS), such as histones or high mobility group box 1 (HMGB1), have been developed as a novel strategy to improve pDNA nuclear importation [5,6]. NLS associated with non-viral cationic vectors can not only improve nuclear importation but also decrease cytotoxicity. As reported previously, PAMAM is an efficient non-viral vector with the ability to rapidly escape from endosomes and release pDNA into cytosol by “proton sponges” [7,8]. Based on our present knowledge of HMGB1-mediated nuclear importation and PAMAM-induced endosomal escape, we believe that PAMAM/HMGB1/pDNA nanocomplexes will be an efficient delivery vector for plasmid DNA.

It has been demonstrated that the nonspecific interactions of cationic polymers with blood components can markedly reduce the half-life and targeting ability of complexes [9]. Cationic

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polymer/DNA complexes have a disappointingly short blood circulation time with rapid hepatic uptake and urinary excretion of partially degraded DNA, resulting in a lower targeting effect for the intended tissue especially in tumors [10]. Attempts to targeting delivery of DNA by application of vectors modified with polyethylene glycol (PEG) and ligands are now receiving considerable attention [11–13]. In a previous study, folate-mediated targeting of FR-positive tumor cells has made dramatic progress [14]. However, modification of PAMAM with PEG or folate using covalent linkage strongly inhibits the ability to condense DNA and to escape from endosomes, which results in a significantly reduced expression efficiency [15].

Recently, pH-sensitive anionic biomaterials coated polymer/DNA complexes have been developed as an efficient non-viral gene delivery system [16]. Carboxylated chitosan is a pH-sensitive and negatively charged biomaterial which can self-assembly complex with cationic polymers through electrostatic interaction. Coating with carboxylated chitosan on the surface of cationic polymer/DNA nanocomplexes has several advantages. Firstly, carboxylated chitosan lowers the ζ-potential of the complexes, avoids albumin-induced aggregation in the blood [17] and reduces the cytotoxicity of cationic polymers [18]. Secondly, the pH-sensitive carboxyl groups on carboxylated chitosan could become protonated in acidic endosomes and dissociate from cationic polymers [19]. Therefore, attempts to modify carboxylated chitosan with PEG and folate ligand could affect the tissue distribution and promote tumor site accumulation while, at the same time, producing a high transfection and expression efficiency of DNA.

In this study, we developed a gene delivery system consisting of PAMAM/HMGB1/pDNA nanocomplexes (PHDs) as the core and a pH-sensitive anionic polymer folate-modified polyethylene glycol tethered carboxylated chitosan (FA-PEG-CCTS) coating on the surface of the PHDs, forming a multifunctional vector FA-PEG-CCTS/PAMAM/HMGB1/pDNA nanocomplexes (FPCPHDs). The FA-PEG-CCTS served as a homing device targeting the tumor and a pH-sensitive biomaterial, in an attempt to increase the cellular uptake in FR-positive cells and dissociate from PAMAM/HMGB1/pDNA ternary nanocomplexes upon entry into endosomes. HMGB1 was used as a nuclear locating signal to achieve high nuclear importation and gene expression. A schematic diagram depicting the formation of FPCPHDs nanocomplexes and the extracellular and intracellular trafficking is shown in Fig. 1. The stability, DNA condensation and protection efficiency, particle size and ζ-potential, in vitro cytotoxicity and transfection as well as the intracellular trafficking of FPCPHDs were all investigated in detail. Moreover, the competitive inhibition effect of free folic acid was examined in FR-positive KB cells and the effect of in vivo targeting on tumor cells was evaluated using S180 xenograft nude mice. FPCPHDs are expected to be an effective delivery system for plasmid DNA to improve transfection and expression efficiency.

2. Materials and methods

2.1. Materials

PAMAM (G4.0), a high mobility group box 1 (HMGB1) and a hetero-functional PEG derivative (NH_2-PEG-COOH) with an average molecular weight of 3 kDa were purchased from Sigma–Aldrich (St. Louis, MO, USA). Carboxylated chitosan (CCTS, MW 50 kDa, carboxylation ≥60.0%) was obtained from Amresco (Solon, USA). Folate-deficient RPMI 1640 growth medium, fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco (USA). Endo-free Plasmid mini kits were purchased from Omega Bio-Tek (USA). Modified BCA protein assay kits were obtained from Sangon Biotech Co., Ltd. (Shanghai, China) and used as described in the product information sheet. Agarose and ethidium bromide were obtained from Biowest and Invitrogen Corporation, respectively. Dimethyl sulfoxide (DMSO), 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxy succinimide ester (NHS), triethylamine (TEA), fluorescein isothiocyanate (FITC), folic acid (FA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich. Lyso-Tracker-Red and Hoechst 33258 were obtained from the Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). All other chemicals were commercially available reagents of at least analytical grade.

2.2. Plasmids

Reporter plasmids pDsRed-M-N1 and pGL3-Control were used in this study. Plasmid pDsRed-M-N1 was a gift from Tsinghua University and amplified in competent high-copy *Escherichia coli* JM109 strain cells grown in LB medium containing 50 μg/ml kanamycin. pGL3-Control vector was obtained from Promega (WI, USA) and amplified in *E. coli* JM109 strain cells grown in LB medium containing 100 μg/ml ampicillin. Plasmids were collected and purified using an Endo-free Plasmid mini Kit according to the manufacturer's protocol. The quantity and quality of the purified plasmid was assessed by measuring its optical density at 260 nm and 280 nm.

2.3. Synthesis of carboxylated chitosan derivatives

Methoxy polyethylene glycol tethered carboxylated chitosan (PEG-CCTS) and polyethylene glycol tethered carboxylated chitosan modified with folic acid (FA-PEG-CCTS) were synthesized as described previously [20–22]. Briefly, mPEG (3 kDa, 1 mmol), succinic anhydride (10 mmol) and pyridine (0.1 ml) were dissolved in 20 ml chloroform and stirred at 65 °C for 24 h. After filtration, the solvent was removed by evaporation under vacuum. The residue was dissolved in 0.1 M $NaHCO_3$ and extracted with dichloromethane (DCM), followed by precipitation with diethyl ether. mPEG-COOH (1.0 mmol) was activated with NHS (1.2 mmol), EDC (1.2 mmol) and triethylamine (5 mmol) at room temperature in DCM for 12 h mPEG-NHS and carboxylated chitosan (CCTS) were dissolved in water and stirred for 72 h at room temperature. The product was lyophilized after dialyzing (MWCO 14 kDa) against water.

FA-PEG-CCTS was synthesized with folic acid, COOH-PEG_{3000}-NH_2 and CCTS. Folic acid (1 mmol), NHS (1.2 mmol), EDC (1.2 mmol) and triethylamine (5 mmol) were dissolved in anhydrous DMSO and stirred at room temperature for 4 h. FA-NHS was purified by precipitation in cool ethyl ether. COOH-PEG_{3000}-NH_2 (1.0 mmol) and FA-NHS (5.0 mmol) were dissolved in DMSO and stirred for 48 h. FA-PEG-COOH was also activated with NHS/EDC/TEA (molar ratio described above) in DMSO and FA-PEG-NHS was purified by precipitation in cool ethyl ether. FA-PEG-NHS and CCTS were dissolved in 10 ml water and stirred at room temperature for 72 h. The final product, FA-PEG-CCTS, was purified by dialysis (MWCO 14 kDa) against deionized water for 48 h and lyophilization. The synthetic pathways are illustrated in Fig. 2. PEG-CCTS and FA-PEG-CCTS were characterized by FTIR and ^{1}H NMR.

2.4. Acid–base titration

The buffering capacity of PEG-CCTS and FA-PEG-CCTS from pH 7.4 to 5.1 was determined by acid–base titration. Briefly, carboxylated chitosan derivatives were dissolved in 0.01 M NaCl (4 mg/ml) and the solution was adjusted to pH 12 with 1 M NaOH. The diluted solution was titrated by the stepwise addition of 0.01 M HCl to obtain the titration profile. The pKa of PEG-CCTS and FA-PEG-CCTS was determined by the primary derivative method and the buffering capacity was defined as the percentage of amino groups from pH 7.4 to 5.1 as previously described [23,24].

2.5. Preparation of FPCPHDs nanocomplexes

FPCPHDs nanocomplexes were prepared with FA-PEG-CCTS and PAMAM/HMGB1/pDNA ternary complexes (PHDs) at a series of N/P ratios (molar ratio of PAMAM-nitrogen atoms to pDNA-phosphate). The final concentration of pDNA was 1 mg/ml. In brief, plasmid was mixed with HMGB1 (weight ratio of HMGB1 to pDNA 1) and then condensed with PAMAM at various charge ratios, followed by incubation for 30 min at room temperature [25]. FA-PEG-CCTS dissolved in deionized water was added and the mixture was incubated for 30 min at ambient temperature. PAMAM/pDNA (PDs), PAMAM/HMGB1/pDNA (PHDs), CCTS/PAMAM/HMGB1/pDNA (CPHDs) and PEG-CCTS/PAMAM/HMGB1/pDNA (PCPHDs) nanocomplexes were prepared as reference formulations.

2.6. Hoechst 33258 intercalation assay

The DNA condensation with PAMAM, HMGB1 and FA-PEG-CCTS was analyzed using a Hoechst 33258 intercalation assay. Nanocomplexes (500 ng of pDNA) were mixed with 100 μl Hoechst 33258 (0.15 $\mu g \cdot ml^{-1}$) and incubated for 10 min at 37 °C. Then, the fluorescence was measured at 353 nm (ex) and 457 nm (em). The fluorescence of free DNA was used as a control. The encapsulation efficiency was calculated from the following equation: