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Research paper

Chitosan microparticles containing plasmid DNA as potential oral gene delivery system

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Abstract

The potential of chitosan as a polycationic gene carrier for oral administration has been explored since 1990s. Chitosan has been shown to effectively bind DNA in saline or acetic acid solution and protect DNA from nuclease degradation. In this study, pDNA (plasmid DNA) was encapsulated in chitosan microparticles. Chitosan–DNA microparticles were prepared using a complex coacervation process and stability of plasmid DNA was investigated in this complex. The chitosan–DNA microparticles could protect the encapsulated plasmid DNA from nuclease degradation. Release of pDNA from microparticles was studied in simulated gastric, simulated intestinal medium and acidic PBS (phosphate buffer saline) (pH 4.5) buffer at 37 °C, and released pDNA was assayed spectrophotometrically. In vitro release of pDNA from chitosan microparticles was dependent on pH, as the pH of the release medium increased release profile decreased. In in vivo-animal studies blue color was observed with X-gal (4-chloro-5-bromo-3-indolyl-β-galactosidase) staining of histological stomach and small intestine sections after oral administration of pDNA–chitosan microparticles as an indicator of exogeneous gene expression.

Keywords: Chitosan; Microparticles; Gene therapy; Oral gene delivery; Plasmid DNA; Beta-galactosidase expression

1. Introduction

The development of appropriate vehicles to deliver new biological macromolecules is a new challenge for pharmaceutical scientists. For efficient gene delivery, plasmid DNA must be introduced into targeted cells, transcribed and the genetic information ultimately translated into corresponding protein [1]. Although viral gene delivery systems yield high transfection efficiency over a wide range of cell targets [2], they have major drawbacks, such as virally induced inflammatory responses and oncogenic effects [3].

To overcome these disadvantages, gene delivery studies have also been planned for development of non-viral gene delivery systems. They can all be administered repeatedly with minimal host immune response, and are stable in storage, targetable, and easily prepared [4]. Cationic polymers have been shown as promising carriers among the non-viral gene delivery systems [5]. Compared with other non-viral gene delivery systems, polycation-DNA complexes are generally more stable. A number of cationic polymers have been investigated as gene carriers [6–8]. An ideal polymeric gene carrier should have high efficacy of gene transfer, targeting ability and good biocompatibility. High stability, especially in the lyophilized form, is also thought to be an important feature to apply genes as pharmaceuticals [9].

Transfection is hindered by (A) targeting the delivery system to the target cell, (B) transport through the cell membrane, (C) degradation in endolysosomes, and (D) intracellular trafficking of plasmid DNA into the nucleus [1]. Chitosan–pDNA complexes appeared to have ability to overcome three major obstacles for transfection, i.e. cell uptake, endosomal release and nuclear localization [10]. Besides many nanoparticle studies [8,9], plasmid DNA–chitosan complexes were also studied in the form of self aggregates [11,12], emulsions [13] and microspheres [14] for transfection of eucaryotic cells showed that the chitosan can be used in many forms as a promising gene carrier.

In recent years, the potential of chitosan as a polycationic gene carrier has been explored in several research groups [8]. Chitosan [β(1–4)2-amino-2-deoxy-D-glucose] is obtained by

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alkaline deacetylation of chitin. Chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine [15,16]. Chitosans differ in degree of *N*-acetylation (40–98%) and molecular weight (50–2000 kDa) [17]. Mucoadhesive property of chitosan potentially permit a sustained interaction of the macromolecule to be 'delivered' with the membrane epithelia, promoting more efficient uptake [18]. Chitosan is a biodegradable and non-toxic polysaccharide [19]. Due to its good biocompatability and toxicity profile, it has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material for artificial skin and wound healing bandage applications [20].

Chitosan was first described as a delivery system for plasmids by Mumper et al. [21]. In the other examples, small plasmid/chitosan nanoparticles (200–300 nm) were prepared by complex coacervation method by Roy et al. [8]. Mao et al. [9] modified chitosan nanospheres with transferrin and PEG, but no significant enhancement in transfection efficiency was observed.

The oral delivery of peptide, protein, vaccine and nucleic acid-based products is the great challenge in the drug delivery industry. Oral delivery is attractive due to the factors such as ease of administration, and improved patient convenience and compliance, thereby reducing overall healthcare costs.

Chitosan has been used successfully to deliver a reporter gene (encoding chloramphenicol acetyl transferase) orally to enterocytes, Peyer's patches and mesenteric lymph nodes [22]. In another study mice fed with chitosan–DNA particles by mixing their fed, expressed the reporter gene *lacZ* (encoding bacterial β-galactosidase) 5 days after oral administration [8]. This study also showed that orally administered chitosan–DNA complexes can stimulate an immune response to the principal peanut allergen Arah-2.

In this study, we have prepared chitosan–pDNA microparticles and examined in vitro stability and in vivo efficiency of encapsulated DNA. Release profiles of DNA from chitosan–pDNA microparticles in simulated gastric, simulated intestinal medium and acidic PBS buffer were observed. In vivo transfection of chitosan microparticles containing plasmid DNA was investigated. To assess the expression and distribution of transduced genes after oral DNA delivery, pDNA chitosan microparticles were applied to mice orally with a special intragastric drill. Then mice were fed with standard diet. The expression of Lac Z gene and activity of α-galactosidase enzyme in stomach and small intestine tissues were detected using X-gal solution at acidic and alkaline pH. At acidic pH β-galactosidase endogenous activity, and at alkaline pH β-galactosidase exogenous activity were observed.

2. Materials and methods

2.1. Materials

Chitosan H (MW: 1400 kDa, deacetylation degree is 80%) was purchased from MGF Co. Ltd (Japan). Plasmid pnlacF was kindly contributed by R. Palmiter (Howard Hughes Medical Institute Research Laboratories, University of Washington, USA). pnlacF (6900 bp) contains lacZ gene which encodes β-galactosidase, ampicillin resistance gene and SV 40 early promoter.

Chitosanase and DNase I were obtained from Sigma–Aldrich Corp. (USA); restriction enzymes BamHI and EcoRI were obtained form Promega Corp. (USA); X-Gal (4-chloro-5-bromo-3-indolyl-β-galactosidase) was obtained from Bio-Rad Lab. Inc., (USA); mice (4–6 weeks, 20–25 g) were obtained from Experimental Animals Department of Hacettepe University (Ankara, Turkey). All other chemicals were obtained from Sigma–Aldrich Corp. (USA).

2.2. Amplification and purification of plasmid DNA

pnlacF was amplified in JM 109 strain of *E. coli* and extracted by alkaline-lysis technique [23] and purified by precipitation with ethanol. Then the precipitate was dissolved in TRIS-acetate-EDTA buffer and concentration of DNA was determined UV-spectrophotometrically at 260 nm. The integrity of isolated plasmid DNA was observed by agarose gel electrophoresis (0.8%, w/v).

2.3. Analysis of DNA structure

The DNA structure was analyzed by digesting with BamHI and EcoRI restriction enzymes. The digestion was performed at 37 °C for 4 h. Electrophoretic mobility of the digested DNA was analyzed by 0.8% agarose gel electrophoresis. Samples were run for 60 min at 110 V in TRIS-acetate-EDTA buffer system (pH 8.0) and visualized using ethidium bromide staining.

Transilluminator and imaging system (Alpha-Innotech Corp., USA) were used to detect DNA and to take photographs under UV light.

2.4. Preparation and characterization of chitosan–DNA microparticles

Plasmid loaded chitosan microparticles were prepared by complex coacervation method [14,24]. Sodium sulfate solution (20%, w/v) containing plasmid was dropped into the chitosan solution (0.25% in 0.1 M acetate buffer, pH 4.5) and stirred (Heidoph, Germany) at 700 rpm for 40 min. Formed particles in the suspension were frozen at −20 °C without separation, lyophilized and stored at 4 °C after freeze–drying (Vitris Freezemobile 5). A gel retardation assay was applied for monitoring pDNA–chitosan complex formation ratios by loading samples onto 0.8% agarose gel. After staining in ethidium bromide (EtBr) gel photographs were taken under UV light. Size of microparticles was determined using an ocular micrometer in a light microscope (Leica, Germany). Leica DC 300 digital camera is used and digitalized microscope images displayed on the PC screen. Transmission Electron Microscopy (TEM) was used to determine particle morphology and confirm particle size range. TEM equipped with a 100 kV Jeol JEM-100 CX with 2A resolving power.