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In vitro and in vivo assessment of chitosan modified urocanic acid as gene carrier

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ABSTRACT

Chitosan nanoparticles modified with 10 and 30% urocanic acid (CUA) via carbodiimide crosslinking were examined for an efficient gene delivery carrier. The CUA gene carrier was characterized by FTIR, TEM, SEM and the in vitro transfection efficiency CUA polyplex was tested with HeLa and 3T3 cells. The loading efficiency of CUA complexes with DNA was assessed at different N/P ratio of 1, 2, 4, 6, 8, and 10. The DNA loading efficiency was found be to >85% for chitosan, CUA10 and CUA30% and the DNA protection ability of CUA10 and CUA30 nanoparticle complexes was confirmed upon incubation with *Nhe*I and *Hin*dIII. The cell toxicity and cell viability results have supported the non-toxic nature of CUA10 and CUA30 nanoparticles. In vitro transfection efficiency of CUA10 and CUA30 polyplex was tested for EGFP expression in 3T3 and HeLa cells and a relative maximum % transfection of about 10% was confirmed by CUA10 and CUA30 after 96 h transfection. The feasibility and biocompatibility of CUA gene carrier in transgenic chickens was also demonstrated. The in vitro transfection and in vivo embryonic viability studies further confirmed the CUA as promising gene carrier because of the improved biocompatibility and DNA protection ability.

1. Introduction

The recombinant proteins with pharmaceutical value are highly desirable in large volumes for the treatment of various diseases in humans. A range of such recombinant proteins are expressed in bacterial or mammalian cells and the mass production can be expanded with the aid of fermenter or bioreactors. However, the inadequacy of using bacterial systems is inability to synthesize complex proteins such as monoclonal antibodies or coagulation blood factors and certain proteins produced in bacteria are tend aggregate easily which are difficult to separate without denaturation. Besides, post-translational modification events such as glycosylation, g-carboxylation, phosphorylation, and sulphatation cannot be accomplished with the bacteria [1]. This constraint could be better resolved by yeast expression system, unlike bacterial cells it can synthesize and secrete matured proteins at a greater extend. Since there is no universal expression system, the secretion of active proteases along with the desired protein might possibly affect the yield in yeast strains [2].

Transgenic animals such as cow, pigs, goat or sheep are feasible alternate for the large-scale production of recombinant proteins via the milk, blood, urine, or seminal plasma. Although the recombinant protein purification is less intricate, the presence of endogenous proteins like serum albumin or antibodies should be considered. In addition the existence of prions during the recombinant protein expression is a serious concern [3]. Because of lesser incubation time, reduction of disease risk and an easily controllable environment may represent transgenic chickens for the production of pharmaceutical proteins [4]. Gelatin/calcium phosphate nanoparticles (GCaPs) surface modified with cholaminchloride hydrochloride were successfully used as gene carriers for the development of transgenic chicken [5].

The non-viral gene delivery systems are continually developed at better rate than virus mediated gene transfection since the later method has often suffered with cytotoxicity and immunogenicity hitches. But, still the transfection efficiency/rate in non-viral methods is not as high as viral gene delivery systems. Generally the higher gene transfection efficiency can be attained by the successful delivery of target genes into the cytosol and circumventing the endocytosis pathway. In case of DNA-based gene therapy the exogenous plasmid DNA is delivered to the cellular nucleus, which encodes a specific gene that enhances

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the expression of therapeutic proteins [6]. Polymers and lipids are highly regarded non-viral methods for the delivery of plasmid DNA [7]. The exclusive features of polymeric carriers viz., safe for periodic injection, easy fabrication and cost effectiveness make them as an attraction carrier for the product design and development of pharmaceuticals [8]. A wide range of polymer-based non-viral gene carriers have developed in the past years since they are relatively low in immunogenicity and cytotoxicity and also can accommodate larger DNA [9].

Cationic polymers are immensely studied as gene carriers because it can effectively condense with the negatively charged nucleic acids via electrostatic interaction. Chitosan, gelatin, dextran and cellulose are widely engaged natural cationic polymers for the active gene delivery since they are highly biocompatible and own relatively low cytotoxicity. The utilization of chitosan as an effective polyplex was originally reported in 1996 for the controlled gene delivery to muscle [10]. However, the main limitation of chitosan is its low solubility at a physiological pH which holds its therapeutical application. The primary amine and secondary hydroxyl groups in chitosan polymer chain offers a greater degree of surface modification that could be expected to improve the chitosan solubility. In vitro gene transfection efficiency of chitosan was significantly enhanced by coupling with urocanic acid for plasmid DNA delivery via proton sponge mechanism [11]. The recent advancement in nanoplex formulation is to enhance the gene transfection efficiency facilitated with endosomolytic peptides through pH dependent endosomal membrane disruption [12]. Herein, the in vitro and in vivo transfection efficiency of chitosan nanoparticles modified with 10 and 30% urocanic acid (CUA-10 and CUA-30) as a gene carrier is reported. The CUA gene carrier was extensively characterized by FTIR, TEM, SEM and the in vitro transfection efficiency CUA polyplex was tested with HeLa and 3T3 cells. In addition, the feasibility and biocompatibility of CUA gene carrier in transgenic chickens is also demonstrated.

2. Experimental

2.1. Urocanic acid-modified chitosan (CUA) preparation

In brief, Chitosan (150 kDa) was coupled with different ratio of (10 and 30 wt%) urocanic acid by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). 0.25 g of chitosan was dissolved in 5.0 mL hydrochloric acid (0.5 M) and then treated with 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (0.1 M) to a final volume of 20 mL (pH 5.5). Meanwhile, urocanic acid was activated by *N*-hydroxysuccinimide (NHS) and EDC with the molar ratio of 1:1. The EDC-NHS activated urocanic acid was mixed with the chitosan and the reaction was continued for 24 h under stirring at room temperature. The reaction was terminated by hydroxylamine (10 mM) and the pH of the solution was adjusted to 8.0. The as-prepared urocanic acid-chitosan (CUA) suspension was dialyzed against distilled water (MWCO-12.0-14.0 kDa) for 4 days. The dialyzed CUA solution was centrifuged at 6000 rpm for 10 min and the resulting precipitate was again centrifuged three times before freeze dried. 5 mM acetic acid was generally added to dissolve the lyophilized sample. The free amino groups in CUA complex was determined by ninhydrin assay [13].

2.2. CUA-DNA nanoparticle characterization

For the particle size and surface charge analysis, the CUA-DNA suspension was prepared at the charge ratio (N/P) 1, 2, 4, 6, 8, and 10. The measurements were carried out using a dynamic light scattering and a laser particle size analyzer (Zetasizer-3000HS, Malvern Inst. Ltd., Worcestershire, UK) at 25 °C with a detection angle of 90° and a refraction index of 1.33. The DNA concentration of 15 μg/mL was retained for each sample. The functional groups in CUA nanoparticle was confirmed by Fourier transform infrared spectroscopy (FT/IR-300, JASCO, Japan) at the standard wave number range of 400–4000 cm^{-1}. For TEM, the CUA-DNA sample was stained with aqueous uranyl acetate (1%) for 15 s and examined by a HITACHI TEM H-7500 microscope (HITACHI, Japan) at 120 kV. The morphology of the CUA10-DNA and CUA30-DNA nanoparticles was observed by a field emission scanning electron microscope (JEOL JSM-6300F).

2.3. Restriction digestion of CUA-DNA complex

The *Escherichia coli* BL-21 (E.coli) strain was used for the pEGFP-C1 plasmid construction and the strains were grown in Luria Bertani broth (LB) medium at 37 °C overnight with shaking (250 rpm). The plasmid DNA (*E. coli* BL-21) was isolated and purified by HiSpeed plasmid Maxi Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The restriction profiles of CUA-DNA and the naked DNA (1 μg) were assessed by incubation with the restriction enzymes such as *Nhe*I and *Hin*dIII (250 U/mL) in digestion buffer at 37 °C for 2 h. The digested DNA fragments were examined by 1% agarose gel electrophoresis.

2.4. CUA-DNA nanoparticle preparation and DNA loading efficiency

The CUA-DNA charge ratio (N/P ratio) was defined by the molar ratio of amine groups in CUA vs. the phosphate groups in DNA. As mentioned above, free amino groups in CUA was determined by ninhydrin assay and the CUA-DNA complex was formed by mixing plasmid DNA (100 μg/mL) to the CUA solution (100 μg/mL, and 1000 μg/mL) in 5 mM sodium acetate buffer (pH 5.5) via self-assembly. The N/P charge ratio of 1, 2, 4, 6, 8 and 10 were prepared to assess the loading efficiency. To obtain CUA-DNA nanoparticle, 200 μL of urocanic acid modified chitosan solution was mixed with 200 μL DNA for 30 s under vigorous stirring. Followed by, 100 μL of 0.1 N sodium hydroxyl solution was added to the 1.0 mL of CUA-DNA solution (DNA - 100 μg/mL) and vigorously vortexes for 1 min. The resultant suspension was centrifuged at 14,000 rpm for 20 min and the supernatant was collected and subjected to the DNA quantification. The CUA-DNA complex formation was confirmed by electrophoresis on a 1% agarose gel with Tris-acetate (TAE) running buffer at 100 V for 40 min and the DNA was visualized with ethidium bromide (0.2 μg/mL).

2.5. Cell culture

HeLa and 3 T3 cell lines were used for the in vitro study and the cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% antibiotics (streptomycin at 100 μg/mL, and penicillin at 100 U/mL) and incubated at 37 °C with 5% CO_2. The culture medium was refreshed in every three days.

2.6. Cell cytotoxicity and viability

The biocompatibility of CUA10-DNA (DNA conc. 1.0 μg) and CUA30-DNA (1.0 μg) was assessed on HeLa and 3T3 cells by water-soluble tetrazolium (WST-1) assay and the lactate dehydrogenase (LDH) assay. 5×10^3 cells/well were seeded onto a 96-well plate and incubated with 200 μL of DMEM supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin, amphotericin B solution at 37 °C and 5% CO_2. The cells were co-cultivated with CUA10-DNA, CUA 30-DNA and Lipofectamine™ 2000 and the cell viability was measured using the WST-1 assay ((Roche Diagnostics, Germany) on day 1 and day 3 by an ELISA reader at 450 nm. Similarly, the cytotoxicity of the particle-DNA complex was measured using the commercial LDH assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) according to the manufacturer's instruction (OD at 490 nm) on day 1 and day 3.

2.7. In vitro transfection

For in vitro transfection, HeLa cells and 3T3 cells were seeded onto a 96-well plate at a cell density of 5×10^4 cells/well and cultivated for 24 h