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Lipid coated chitosan-DNA nanoparticles for enhanced gene delivery

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ABSTRACT

Chitosan as a polycationic non-viral vector for gene delivery has the advantage of being a biocompatible and biodegradable polymer. However, without laborious chemical modifications to its structure, it is of limited use as a gene delivery vehicle due to its low ability to efficiently transfect under physiological conditions. To address this problem, we developed novel liposome encapsulated chitosan nanoparticles; lipochitoplexes (LCPs). Chitosan nanoparticles (CsNPs) were obtained using the ionic gelation technique. For this purpose, an ultrapure low molecular weight chitosan with a high degree of deacetylation was cross-linked using polyanionic tripolyphosphate resulting in efficient entrapment of plasmid DNA (pDNA) inside the nanoparticles. LCPs were prepared by incubating chitosan nanoparticles together with anionic liposomes (DPPC/Cholesterol). The LCPs offered better pDNA protection, reduced cytotoxicity and at least twofold increase in the transfection efficiency under physiological conditions. The efficiency of our delivery vehicle was also proved *in vivo* in the chorioallantoic membrane model (CAM). LCPs were able to transfect the CAM without traumatising the surrounding blood vessels. This new biocompatible composite system devoid of chemical modifications, organic solvents and harsh production conditions makes it an optimal gene delivery vehicle for *in vivo* applications offering new insights into the field of non-viral gene therapy.

1. Introduction

Just a few decades ago, gene therapy was highly contemplated and faced much scepticism from the scientific community. After a series of fatal incidents caused due to the use of viral vectors, most of which were due to immunogenic reactions or due to mutations caused by the viruses, gene therapy has faced a strong setback (Hacein-Bey-Abina et al., 2003; Raper et al., 2003). Consequently, all clinical trials for gene therapy came to a standstill, especially those involving viral vectors and their use for gene therapy was being re-evaluated and their role being limited to *ex vivo* applications due to safety concerns. At this point, research into non-viral gene delivery vehicles saw a surge, and scientists began exploiting the already popular cationic lipid based vectors and polymers (Segura and Shea, 2001; Thomas and Klibanov, 2003). Cationic delivery vehicles electrostatically interact with the cell membrane and effectively mimic the viral vectors by gaining cellular entry through various cellular uptake pathways depending upon their formulations (Engelhardt et al., 2017; Khalil et al., 2006). Owing to their ease of use and transfection efficiencies, delivery vehicles such as DOTAP, PEI, poly-l-lysine and chitosan among others have gained widespread interest (Borchard, 2001; Davis, 2002).

Chitosan's ability as a biocompatible and biodegradable polymer for gene delivery was realised during the late nineties during the search for newer non-viral vectors (Murata et al., 1996; Patrick Erbacher et al., 1998). This was followed by chemical and structural modifications of the polymer to enhance its cellular uptake and transfection efficiency under physiological conditions (Germershaus et al., 2008; Haas et al., 2005; Kim et al., 2007) Chitosan's transfection efficiency mainly depends upon its molecular weight and the degree of deacetylation *i.e.* the number of primary amine groups which are protonable in acidic

pH (Koping-Hoggard et al., 2003, 2004). Although chitosan/nucleic acid complexes are promising gene delivery vehicles, under physiological conditions they suffer from poor cellular uptake and endosomal escape (Uchegbu et al., 2008). This is partly due the rapid

Abbreviations: CAM, chorioallantoic membrane; CsNPs, chitosan nanoparticles; DLS, dynamic light scattering; DPPC, dipalmitoylphosphatidylcholine; EDD, egg development day; LCPs, lipochitoplexes; LDV, laser doppler velocimetry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDI, polydispersity index; pDNA, plasmid DNA; PEI, polyethylenimine; TPP, tripolyphosphate

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deprotonation of chitosan in physiological pH which results in partial deformation of the complex, meaning a net decrease in the transfection efficiency with increasing pH. The optimal pH range for chitosan based gene delivery vehicles is between 6.8 and 7.0, which is not suitable for *in vivo* drug delivery (Sato et al., 2001). The other main drawback of the cationic delivery vehicles is their cationic nature *i.e.* when not optimally formulated, they tend to be cytotoxic (Bor et al., 2016; Lv et al., 2006). Addition of a suitable cross-linker to the formulation occupies the unbound primary amines. Cross-linking also provides a better scope for the entrapment of nucleic acids (Gan et al., 2005; Martinez et al., 2007). Tripolyphosphate (TPP), a commercially available polyanion, is commonly employed as a cross-linker for the formation of chitosan-TPP/DNA nanoparticles by ionic gelation technique. As a polyanion, TPP binds to the primary amine groups of chitosan, thereby reducing its surface charge and increasing the structural stability of the complex (Fan et al., 2012; Koukaras et al., 2012) Liposomes and synthetic peptide mimicking lipids have been used for successfully for non-viral gene delivery *in vitro* (Janich et al., 2016; Wölk et al., 2015). Liposomal formulation containing phospholipids (*e.g.* DPPC) and cholesterol offer room for optimisation in terms of surface charge and mechanical stability which have a significant effect on the transfection (Khiati et al., 2009; Zuhorn et al., 2002). Encapsulation of cationic nanoparticles in anionic phospholipid comprising liposomes improves the transfection profile and cytotoxicity profile of the system. This combination has shown promising transfection and knockdown efficiencies *in vitro* (Ewe et al., 2016; Schäfer et al., 2010).

In this study, we employed the aforementioned method of liposomal encapsulation for CsNPs loaded with pDNA resulting in a more efficient gene delivery vehicle. The liposomal shell around the CsNPs protects chitosan from being deprotonated in physiological pH. This encapsulation technique showed increased transfection efficiency and significantly higher safety profile when compared to cationic polymer complexes with PEI or chitosan. The transfection efficiency of LCPs was evaluated both *in vitro* in HEK-293 cells and *in vivo* in the chorioallantoic membrane model. Toxicity of the delivery vehicles was evaluated using the MTT assay and the haemolysis potential of the delivery vehicles was assessed using the haemolysis assay.

2. Materials and methods

2.1. Materials

Ultrapure chitosan of non-animal origin (KiOmedine-CsU® produced from white mushrooms (*Agaricus bisporus*) molecular weight 60 kDa, deacetylation degree 84 mol %), Sodium tripolyphosphate pentabasic (≥98%), Acetic acid (≥99.8%) and Cholesterol (≥99%) were all obtained from Sigma Aldrich (Taufkirchen, Germany). Sodium acetate (≥98.5%), sodium chloride (≥99.5%) were obtained from Carl Roth (Karlsruhe, Germany). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, USA). Branched PEI 25 kDa was a gift from BASF (Ludwigshafen, Germany). Plasmid DNA pCMV-*luc* which encodes for luciferase from the firefly (*Photinus pyralis*) driven by a human cytomegalovirus (CMV) promoter and pCMV-GFP encoding for green fluorescence protein were purchased from Plasmid Factory (Bielefeld, Germany). All other chemicals and reagents used were of analytical grade and all buffers used were prepared in the laboratory. Double distilled water which was autoclaved and filter sterilised using 0.22 µm filters (Whatman, Dassel, Germany) was used for all experiments.

2.2. Cell culture

Human embryonic kidney cells (HEK-293) were purchased from American Type Culture Collection (Manassas, USA). The cells were cultured in DMEM-HG medium (Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 10% foetal bovine serum (Sigma Aldrich) and maintained at 37 °C and 8.5% CO_2 under humid conditions. The cells were cultivated in monolayers and were split upon reaching 80% confluency.

2.3. Preparation of chitosan nanoparticles

Nanoparticles were produced using ionic gelation technique developed by Calvo et al. (Calvo et al., 1997). Nanoparticles preparation method and parameters were investigated and optimised through preliminary experiments. Chitosan solution (0.5 mg/mL) was prepared by dissolving chitosan in sodium acetate/acetic acid buffer solution (0.2 M, pH 4.0) by stirring the solution overnight using a magnetic stirrer at room temperature. TPP solution (0.5 mg/mL) was prepared by dissolving TPP in water. Both chitosan and TPP solutions were filter sterilised prior to use. pDNA was diluted with water to a concentration of 0.5 mg/mL. Specific volumes of TPP and pDNA solutions were mixed together prior to their addition to the chitosan solution. Mass ratios of 3:1 (Chitosan to TPP) and 4:1 (Chitosan to pDNA) were chosen. Chitosan solution was pipetted into a round-bottom flask and stirred using a magnetic stirrer at medium speed (700 rpm). TPP and pDNA mixture was then added to the chitosan solution and an instant nanoparticle formation was confirmed optically by observing a light blue opalescence. The reaction was carried out for 30 min and stored at 4 °C.

2.4. Preparation of liposomes and lipochitoplexes

Liposomes were prepared by adding DPPC and cholesterol (80:20 mol %) dissolved in 2:1 (v/v) Chloroform: Methanol mixture to a 10 mL round bottom flask. A thin lipid film was obtained by evaporating the solvent at 40 °C using a Laborota 400 rotary evaporator (Heidolph Instruments, Schwabach, Germany). 20 mM HEPES buffer (pH 7.4) was added to the lipid film and sonicated in an ultrasound bath to obtain a uniform suspension of liposomes. This was followed by extrusion (21 times) through 400 and 200 nm polycarbonate membranes (Whatman) respectively using an Avanti Mini Extruder (Avanti Polar Lipids) to reduce their size. The liposomes were filtered through 0.22 µm syringe filters prior to their addition to the CsNPs. To optimise the stoichiometry of nanoparticles and liposomes, different CsNP:liposome mass ratios were used. Appropriate amounts of liposomes were added to the CsNPs and mixed vigorously by pipetting. The mixture was incubated for 1 h at room temperature.

2.5. Particle size distribution and ζ-potential

The hydrodynamic diameter of the liposomes, CsNPs and LCPs was analysed by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany). For analysis of the data, viscosity (0.88 mPa.s) and refractive index (1.33) of water at 25 °C were considered. The instrument is equipped with a 10 mW HeNe laser and the measurements were performed at a wavelength of 633 nm and a detection angle of 173° backscatter. Measurement position and laser attenuation were automatically adjusted by the instrument. The instrument was set to perform 15 size runs per measurement each lasting 10 s. Results are expressed as size distribution by number.

The ζ-potential measurements were performed by laser Doppler velocimetry (LDV) using the Zetasizer Nano ZS in a clear disposable folded capillary cell (DTS1060, Malvern Instruments). Depending upon the sample, the instrument automatically performs 15–100 runs per measurement. Three independent formulations were measured for both DLS and LDV analyses.

2.6. Atomic force microscopy (AFM)

AFM was used to determine the morphology of the particles and to confirm the particle size. A small amount of the sample (10 µL) to be analysed was pipetted onto silica wafers which were glued to glass