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Factors influencing the transfection efficiency of ultra low molecular weight chitosan/hyaluronic acid nanoparticles

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ABSTRACT

The present work describes nanoparticles made of ultra low molecular weight chitosan (ULMWCh)/hyaluronic acid (HA) as novel potential carriers for gene delivery. Small and monodispersed nanoparticles with high *in vitro* transfection capabilities have been obtained by the complexation of these two polyelectrolytes. ULMWCh (<10 kDa) presents more advantageous characteristics over the higher molecular weight chitosan for clinical applications, namely increased solubility at physiological pH and improved DNA release. The ULMWCh:HA ratio and the HA molecular weights were varied with the aim of obtaining particles in the 100 nm range. Using chitosan (Ch) with a molecular weight of 5 kDa, HA with a molecular weight of 64 kDa, and a weight ratio of 4:1, nanoparticles with a *Z*-average size of 146 ± 1 nm and narrow size distribution (polydispersity index: 0.073 ± 0.030) were obtained. Nanoparticle images taken in dry conditions by SEM and AFM showed spherical particles. The optimal pH for transfection ranged from 6.4 to 6.8 for 0.25 μg of EGFP plasmid per well, with an incubation time of 4 h. Using these optimized parameters, DNA/ULMWCh:HA nanoparticles successfully transfected $25 \pm 1\%$ of the 293T cells with pEGFP, compared to 0.7% obtained for DNA/ULMWCh under the same conditions. This high transfection efficiency of our non-viral gene delivery system could be attributed to the synergic effect of ULMWCh and low charge density of the HA chain for easy release of DNA which makes the system suitable for targeted gene delivery.

1. Introduction

Many studies have demonstrated that the modification of the specific properties of DNA-embedded matrices can significantly increase *in vitro* gene activity as compared to bolus delivery [1,2]. A simple approach consists in using a cationic polymer such as chitosan (Ch), complexed with DNA via electrostatic interactions. Ch is used for this purpose owing to its advantageous properties, such as its biocompatibility and biodegradable nature [3]. However, numerous factors influence the stability and transfection efficiency of Ch-based systems, including molecular weight and deacetylation level. Particularly, low molecular weight Ch (LMWCh) presents more interesting characteristics for clinical applications. These include increased solubility at physiological pH [4] and improved DNA release [5,6]. Also, LMWCh provides antimicrobial, immunostimulant, antioxidant, and cancer growth inhibitory effects [7]. Conversely, studies report a decreased transfection efficiency when lower molecular weight Ch is used [8–10]. For example, Huang et al. observed a smaller percentage of cells expressing green fluorescent protein (GFP) when transfecting with LMWCh (10 kDa; 0.6%) in contrast with high molecular weight Ch (HMWCh) (213 kDa; 12.1%) [8]. Kiang et al. demonstrated that particles' formation with LMWCh and DNA may not be energetically favorable [11]. In such a system, LMWCh loses the chain entanglement effect found in longer Ch chains, which is partially responsible for the particle stability.

An approach for the improvement of DNA/LMWCh particle stability and transfection efficiency used in this study involves the association of LMWCh with an anionic biopolymer, such as alginate (Alg), prior to the addition of DNA. This allows a more stable polyplex formation and smaller nanoparticles than the ones formed with LMWCh alone [12]. The choice of the anionic polymer, however, could greatly influence the specificity, stability and size of the assembled nanoparticles. Hyaluronic acid (HA) is another biocompatible anionic biopolymer naturally found in humans and

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is used for a great number of clinical applications [13,14]. This biopolymer has some advantageous properties when compared to Alg, including its ability to bind various cellular receptors such as CD44 [15], which is expressed in normal human cells and in cancerous cells [16,17]. The targeting of the gene carriers is of particular interest for cancer therapy [18], as this would increase the efficiency [19] and reduce side effects compared to non-targeted treatments such as death of healthy tissues.

In vitro and *in vivo* experiments have demonstrated that Ch/HA micro sized particles can be used as carriers in drug release systems [20,21]. Recent studies have also indicated that anti-cancer medicines conjugated to HA increase the specificity of the drug for cancerous cells via a specific HA/CD44 receptor interaction [22]. However, HA requires an association with a cationic polymer in order to effectively transfect cells with DNA. Previously, Mok et al. [23] demonstrated the advantage to prepare nanoparticles via assembly of a complex containing HA and protamine (a polycation), which allowed the specific release of siRNA into the cells. Despite this interesting result, the use of protamine for gene therapy is not ideal as it may elicit an immune response [24], which is unlikely to occur when using Ch.

Recently, De La Fuente et al. reported the possibility of using LMWCh with a molecular weight of 10 kDa, complexed with HA for *in vitro* gene delivery [25]. However, the transfection level was low for this system. Such issue may be related to the molecular weight of Ch used which is still believed to be high for this combination. Indeed, Koping-Hoggard et al. have shown that a small change in Ch molecular weight between 1.2 and 10 kDa could greatly alter the transfection efficiency [5]. While ULMWCh could have a great potential for *in vitro* and *in vivo* gene delivery, the weaknesses of a Ch delivery system is the need for an excess of ULMWCh (+/− charge ratio of 60:1) to stabilize the polyplex in addition to a lack of targeting ability of the Ch.

In this work, we hypothesize that the use of ULMWCh (5 kDa) complexed with low charge density polyanionic polyelectrolyte such as HA produces non-viral nanocarriers with high transfection efficiency. ULMWCh presents more adapted properties for transfection than LMWCh (10 kDa) such as better solubility and lower hepatotropic behavior [4,26], while the presence of HA will provide stability to the system combined with the ability to target specific cells.

The nanoparticles were prepared through the development and optimization of experimental protocols. Their size and charge were determined by dynamic light scattering (DLS) and zeta potential measurements respectively. The morphology of the nanoparticles was imaged with atomic force microscopy (AFM) as well as by scanning electron microscopy (SEM). The transfection efficiency of the system was determined by fluorescent microscopy and fluorescence-activated cell sorting analysis (FACS) using HEK 293T cells.

2. Materials and methods

2.1. Materials

Ch with a molecular weight of 5 kDa and a deacetylation degree of 90% was purchased from Medipol (CH). The polymer HA was obtained from Lifecore Biomedical (USA), with molecular weights of 17, 35 and 64 kDa. The plasmid, pEGFP was supplied by Clontech (CA), the cell line HEK 293T was obtained from ATCC (CA), and lipofectamine™ was purchased from Invitrogen (CA).

2.2. Nanoparticle preparation

A simple method was developed for the preparation of nanoparticles and the incorporation of DNA. ULMWCh was briefly dissolved in 0.01 M hydrochloric acid at a concentration of 1% (w/v), pH 5.5, and HA was dissolved in water at 0.1% (w/v) (pH 5.5). Both solutions were filtered through a 0.2 μm polyethersulfone (PES) membrane (Millipore; CA) and mixed overnight under stirring.

Using the following ULMWCh:HA weight ratios: 1:1; 1:2; 1:3; 1:4; 1:5; 1:6; 1:7, a constant concentration 11.25 μg/mL of HA and a ULMWCh concentration of 11.25; 22.5; 33.75; 45; 56.25; 67.5; 78.75 μg/mL were used, respectively.

2.3. Size, polydispersity and zeta potential measurements

Dynamic light scattering (DLS) (high performance particle sizer (HPPS), Malvern instruments, UK) and Zeta PALS (Brookhaven Instruments, USA) were used to measure size and charge of the nanoparticles respectively. The nanoparticles were prepared and analyzed in distilled water at 25 °C. The value of the *Z*-average was used to determine the hydrodynamic diameter of the nanoparticles using the Stokes–Einstein equation. The polydispersity index (PDI) was also obtained by DLS using the Dispersion Technology and Light Scattering System HPPS software v 4.20.

2.4. Scanning electron microscopy (SEM)

First, nanoparticles were dropped on a silica surface pre-coated with a thin layer of gold and palladium. The nanoparticles were lyophilized using Modulyo D-115 (Thermo Savant) prior to analysis and micrographs were obtained using a FEG-SEM (Hitachi S4700, Japan).

2.5. Atomic force microscopy (AFM)

The same samples used for the FEG-SEM observation were analyzed by the AFM. Images were produced with a Nanoscope III (Digital Instruments, USA) using a silicon cantilever in tapping mode and analyzed using nanoscope v 5.12r5 software.

2.6. DNA extraction and loading

Enhanced green fluorescent protein plasmid (pEGFP; 4.7 kb), driven by a human cytomegalovirus promoter (CMV), was amplified in DH5α and selected for its kanamycin resistance. The DNA was then purified using a Plasmid Maxi kit (Qiagen, CA) following the guidelines provided by the manufacturer. Plasmid DNA (pDNA) was dissolved in water, and the concentration was evaluated using a UV spectrophotometer at 260 nm.

The incorporation of DNA within the polymer complex was performed through electrostatic interactions between the DNA and the ULMWCh. This simply involved incubating the DNA for 15 min with ULMWCh:HA nanoparticles in PBS (concentrations and pH indicated in Fig. 3) prior to the transfection assays.

2.7. Cell culture and transfection

The HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen, USA), at 37 °C, under a 5% CO_2 atmosphere. A subculturing ratio of 1:8 was used when the cells reached 80% of confluence.

Cells were plated at 50,000 cells per well and 500 μL of media in clear 24 well tissue plates (Corning, USA). After incubation for 24 h the medium was discarded, and the cells were washed once with PBS with the same pH that was used for transfection. Nanoparticles containing DNA solution were added to the cells and incubated for a period of 4 h, except as otherwise noted. After this process, the nanoparticle DNA solution was discarded and fresh culture media, supplemented with serum and antibiotics, were added to the cells.

Lipofectamine was used as a positive control for transfection, added in DMEM without serum and antibiotics, following manufacturer procedures. The solutions were incubated for a period of 4 h, with a concentration of 0.25 μg/well of pEGFP, after which the medium was discarded and replaced with a complete medium containing serum and antibiotics.

2.8. Fluorescent microscopy

Fluorescent microscope pictures were taken 48 h after transfection. An inverted microscope (TE2000-U, Nikon; USA) equipped with a high pressure mercury lamp (C-SHG1, Nikon; USA), a digital camera (DXM1200F, Nikon; USA) and the appropriate set of filters for fluorescein (FITC) excitation and emission wavelengths were used. The ACT-1 software was used to acquire pictures of the transfected cells.

2.9. Fluorescence-activated cell sorting analysis (FACS)

The cells were transfected and incubated at 37 °C for 48 h before analysis for GFP expression. Cells were then washed with PBS and incubated with 0.05% trypsin–EDTA (Invitrogen, USA) until cells detached from the surface. After incubation, 1 mL of complete medium was added to inhibit trypsin, followed by centrifugation and washing with PBS (at physiological pH). They were then replenished with PBS containing 2% serum and 3 μM of PI (Propidium iodide, Invitrogen, USA). Transfection efficiency was assessed by FACS analysis with the aid of a FACScalibur (BD Biosciences, CA), which used the 488 nm line of an argon laser to excite both PI and GFP. After the observation under fluorescent microscopy, cell samples were transferred to