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Self-assembled nanoparticles based on amphiphilic chitosan derivative and hyaluronic acid for gene delivery

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ABSTRACT

The present work described nanoparticles (NPs) made of oleoyl-carboxymethy-chitosan (OCMCS)/hyaluronic acid (HA) using coacervation process as novel potential carriers for gene delivery. An N/P ratio of 5 and OCMCS/HA weight ratio of 4 were the optimal conditions leading to the smallest (164.94 nm), positive charged (+14.2 mV) and monodispersed NPs. OCMCS-HA/DNA (OHD) NPs showed higher in vitro DNA release rates and increased cellular uptake by Caco-2 cells due to the HA involved in NPs. The MTT survival assay indicated no significant cytotoxicity. The transfection efficiency of OHD NPs was 5-fold higher than OCMCS/DNA (OD) NPs; however, it decreased significantly in the presence of excess free HA. The results indicated that OHD NPs internalized in Caco-2 cells were mediated by the hyaluronan receptor CD44. The data obtained in the present research gave evidence of the potential of OHD NPs for the targeting and further transfer of genes to the epithelial cells.

1. Introduction

Gene delivery is a promising strategy as the encoded protein can be expressed in the host in its natural form (without denaturation or modification), and caused prolonged expression of the protein (Mao, Sun, & Kissel, 2010) The gastrointestinal (GI) system is an excellent target for noninvasive localized drug delivery due to its large surface area and its accessibility. For this reason, the GI tract has been considered as an attractive target for gene therapy interventions (Nandedkar, 2009). The success in gene therapeutic strategies depends on an efficient system for the delivery of nucleic acid into the target cells (Vadolas, Williamson, & Ioannou, 2002). In recent years, non-viral vectors and especially those resulting from the use of nanotechnologies have received increasing attention for achieving the delivery of genetic material to the GI tract (Luten, van Nostrum, De Smedt, & Hennink, 2008).

Cationic polymers have been shown as promising carriers among the non-viral gene delivery systems. Many cationic polymers, such as chitosan, polylysine, polyethyleneimine, dendrimers, poly (a-(4-aminobutyl)-L-glycolic acid) as well as cationic liposomes have been investigated for gene delivery (Mohammadi et al., 2011). Chitosan has been investigated as nonviral vector for gene delivery because of its ability to condense gene into nanoparticles that are appropriate to be endocytosed by cells, and subsequently be released from endosomes and enter nucleus (Muzzarelli, 2010a). Furthermore, chitosan is an ideal candidate for oral DNA delivery due to its good biocompatibility and high positive charge density conferring it mucoadhesive properties (Lai, Wang, & Hanes, 2009; Muzzarelli, 2010b).

However, chitosan shows two major disadvantages: one is poor solubility because the amino groups on chitosan are only partially protonized at physiological pH 7.4. The other is low transfection efficiency (Gao et al., 2008). In our previous research, oleoyl-carboxymethy-chitosan (OCMCS) has been synthesized and proposed as one of water-soluble chitosan derivatives over a wide pH range (Liu, Cheng, et al., 2012; Liu, Zang, et al., 2012). The transfection efficiency of chitosan vectors can be improved by combining chitosan with cationic or anionic biopolymers, such as polyethyleneimine (Zhao et al., 2009) or arginine (Gao et al., 2008), prior to the addition of DNA. The choice of biopolymer greatly influences the specificity, stability, and size of the assembled nanoparticles (Duceppe & Tabrizian, 2009). Hyaluronic acid (HA) is another biocompatible anionic biopolymer naturally found in humans and is used for a great number of clinical applications (Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). This biopolymer has some advantageous properties, including its ability to bind various cellular receptors such as CD44 (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990), which is expressed in normal human epithelium cells, chondrocytes and cancerous cells (Marhaba & Zoller, 2004). The targeting of the gene carriers

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is of particular interest for oral therapy (Merdan, Kopecek, & Kissel, 2002), as this would increase the efficiency (Li & Huang, 2006) and reduce side effects compared to non-targeted treatments such as death of healthy tissues.

Our goal in the present work was to evaluate the potential of a noval gene nanocarrier, consisting of OCMCS and HA, specially designed for the specific targeting and intracellular delivery of genes into the intestinal epithelial cells.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 50 kDa, degree of deacetylation = 93.15%) was made from crab shell and obtained from Laizhou Haili Biological Product Co., Ltd. (Shandong China). Hyaluronic acid, with a molecular weight of 5 kDa, was purchased from C.P. Freda Pharmaceutical Co. Ltd. (Shangdong, China).

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, OH).

Lipofectamine™ 2000 was obtained from Invitrogen (USA). Plasmids (pEGFP-N2, 4.7 kb) containing a CMV promoter and an enhanced green fluorescence protein reporter were obtained from BD Biosciences Clontech (Palo Alto, CA, USA). pEGFP-N2 was amplified and isolated using a Plasmid Mega Kit (QIAGEN, Valencia, CA, USA). The recovered plasmids were stored at 4 °C in sterile deionized (DI) water. The purity of plasmids was analyzed by gel electrophoresis (0.8% agarose), while their concentration was measured by UV absorption at 260 nm (V-530, Jasco, Tokyo, Japan).

2.2. Preparation of OCMCS/DNA (OD) NPs and OCMCS-HA/DNA (OHD) NPs

Oleoyl-carboxymethy-chitosan (OCMCS) was synthesized by reaction of chitosan with chloroacetic acid and oleoyl chloride as described in our previous study (Li et al., 2006). Solutions of OCMCS at different concentrations were prepared in phosphate buffer saline (PBS, pH 6.5) in order to achieve N/P ratios of 0.5, 1, 2, 5, 10, 15, and 20 (an amino group to a phosphate group ratio hereafter was defined as charge or N/P ratio). A DNA solution of 50 μg/ml in 25 mM of sodium sulfate was prepared. An equal volume of chitosan solution (from each concentration) and DNA solution were added (by dropping slowly) together while stirred at various speed (100–2000 rpm). The final volume of the mixture in each preparation was limited to below 500 μl in order to yield uniform NPs.

OHD NPs were prepared according to the method described by Duceppe and Tabrizian (2009). In brief, 10 mg HA was dissolved in 4 ml of distilled water under magnetic stirring. The OCMCS solution was stirred at a rate of 3000 rpm for 30 min, mixed into the HA solution and stirred for 10 min. Seven different mixtures were prepared with CS:HA weight ratios at 1:1, 2:1, 3:1,4:1, 5:1, 6:1, and 7:1. The required volume of 50 μg/ml plasmid DNA was gently added to OCMCS/HA solution to form complexes of a selected N/P ratio. The mixture was vortexed rapidly for 3–5 s and left for 1 h at room temperature for the complexes to completely form.

2.3. Particle size, zeta potential and surface morphology

The Z-average hydrodynamic diameter and surface charge of NPs were determined by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at room temperature. The NPs were prepared and analyzed in 1 ml distilled water at 25 °C. All samples were measured in triplicate. The morphological characterizations of the NPs were viewed using scanning electron microscopy (SEM, JSM-330A, Japan) and atomic force microscopy (AFM, Dualscope C26, DME, Denmark).

2.4. Gel retarding analysis

OHD NPs were evaluated by agarose gel electrophoresis. The NPs and the naked plasmid were loaded onto a 1% agarose gel containing ethidium bromide in Tris–borate EDTA buffer at pH 8.0. The samples were run on the gel at 120 V for 30 min. The gel was then photographed using a GDS-8000 (UVP, USA).

OHD NPs (N/P ratio 5) prepared with OCMCS: HA weight ratios of 4:1 and naked DNA solution (each containing 1 μg of DNA) were added to 10 U of *Not*I and 15 U *Hin*dIII (Sigma–Aldrich, USA) and kept in a water bath for 30 min at 37 °C. In addition, different concentrations (56–336 ng/μl) of chitosanase (from streptomyces species, activity 18 units/mg, Sigma–Aldrich, USA) were added and the NP–chitosanase solution was maintained in a water bath for 4 h at 37 °C. Agarose gel (1%) electrophoresis was repeated, as described above.

2.5. Stability of OHD NPs

OHD NPs suspension (1 mg/ml, 1 ml) was placed into a cellulose membrane dialysis tube (molecular weight cutoff 8000–10,000). The tube was introduced into the different kinds of simulated fluid referred to Mohammad Reza (Saboktakin, Tabatabaie, Maharramov, & Ramazanov, 2011) and kept in a water bath at 37 °C. At predetermined time intervals (between 0 h and 6 h), whole medium was removed and replaced by fresh simulated fluid. The protocol of using the simulated fluids at different pH was as follows:

- *0–2 h*: simulated gastric fluid, pH 1.2
- *2–4 h*: simulated duodenum fluid, pH 6.0
- *4–6 h*: simulated intercellular spaces of enterocytes fluid, pH 7.4

Simulated gastric fluid (SGF, pH 1.2) consisted of NaCl (0.2 g), HCl (7 ml) and pesin (3.2 g); pH was adjusted by NaOH to 1.2 ± 0.5. Simulated intestinal fluid (SIF) consisted of KH_2PO_4 (6.8 g), 0.2 N NaOH (190 ml), and pancreatin (10.0 g). SIF was adjusted by NaOH to 7.4 ± 0.1 to obtain simulated intercellular spaces of enterocytes fluid (SISEF). Simulated duodenum fluid (SDF) pH 6.0 was prepared by mixing SGF pH 1.2 and SISEF pH 7.4 in a ratio of 30:70. At predetermined time intervals (0 h, 2 h, 4 h and 6 h), the particle size was evaluated via a Zetasizer (Malvern Instruments, UK) and the morphology of the OHD NPs was observed by transmission electron microscope (100 CX II, Japan).

2.6. Evaluation of DNA loading and release

The OD NPs and OHD NPs (N/P ratio 5 and OCMCS/HA weight ratio 4) were formed according to the method described in Section 2.2. After complex was completely formed at ambient temperature for 30 min, the turbid solution was centrifuged at 20,000 × *g* at 4 °C for 30 min and the supernatant was collected to determine the amount of free DNA. The loading efficiency (LE %) of DNA was calculated by Eq. (1), and the analyses were performed in triplicates.

$$\text{LE\% of DNA} = \left[\frac{A-B}{A}\right] \times 100\% \quad (1)$$

where *A* is the total concentration of DNA (mg/ml) and *B* is the concentration of unloaded DNA (mg/ml).

Phosphate buffer saline (PBS, pH 7.4) was used as media for the DNA release study. Dried OD NPs and OHD NPs (3.9 mg) and buffer solution (1.2 ml) were placed in a micro tube and incubated in a