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Targeted delivery of small interfering RNA to colon cancer cells using chitosan and PEGylated chitosan nanoparticles

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ABSTRACT

Small interfering RNA (siRNA) molecules specifically target messenger RNA species, decreasing intracellular protein levels. β-Catenin protein concentrations are increased in 70–80% of colon tumors, promoting tumor progression. Chitosan exhibits low levels of toxicity and can be transported across mucosal membranes; therefore, our objective was to develop chitosan and poly(ethylene glycol)-grafted (PEGylated) chitosan nanoparticles, 100–150 nm in diameter, encapsulating anti-β-catenin siRNA for transfection into colon cancer cells. Encapsulation efficiencies up to 97% were observed. Confocal microscopy visualized the entry of fluorescently-tagged siRNA into cells. Western blot analysis showed that both chitosan and PEGylated chitosan nanoparticles containing anti-β-catenin siRNA decreased β-catenin protein levels in cultured colon cancer cells. These results indicate that nanoparticles made with chitosan and PEGylated chitosan can successfully enter colon cancer cells and decrease the level of a protein that promotes tumor progression. These or similar nanoparticles may prove beneficial for the treatment of colon cancer in humans.

1. Introduction

Chitosan is a naturally occurring cationic carbohydrate polymer comprised of glucosamine and *N*-acetylglucosamine residues. Its basic amine groups are protonated at physiological pH, exhibiting interesting biopharmaceutical properties. Specifically, chitosan has low levels of immunogenicity (Agnihotri, Mallikarjuna, & Aminabhavi, 2004) with minimal toxicity, depending on its molecular weight and degree of deacetylation (Akhtar & Benter, 2007; Garcia-Fuentes & Alonso, 2012). Chitosan can interact with negatively charged epithelial surfaces or with mucosal surfaces through electrostatic interactions with charged sugar groups like sialic acid (Aagaard & Rossi, 2007; Casettari et al., 2012; Ganguly, Chaturvedi, More, Nadagouda, & Aminabhavi, 2014; He, Davis, & Illum, 1998). Importantly, chitosan can be effectively transported across mucosal membranes due to a combination of bioadhesion (delayed clearance from the site of absorption) and transient opening of tight junctions between epithelial cells in the mucosal membrane (Casettari et al., 2012). Because pH changes throughout the gastrointestinal tract and electrostatic interactions are crucial for maintaining the stability of nanoparticles, chitosan's permanent positive charges favor mucoadhesion. Previously, small interfering RNA (siRNA) loaded nanoparticles of chitosan and poly(ethylene glycol)-grafted (PEGylated) chitosan have been prepared by self-assembly via electrostatic interactions between the negatively charged drug and the positively charged chitosan (Rudzinski & Aminabhavi, 2010). Due to these advantages, the ability of chitosan-based nanoparticles to deliver siRNA has been examined by our group and others (Buschmann et al., 2013; Katas & Alpar, 2006; Lai & Lin, 2009; Rudzinski & Aminabhavi, 2010; Vauthier, Zandanel & Ramon, 2013). This property has been exploited here to develop

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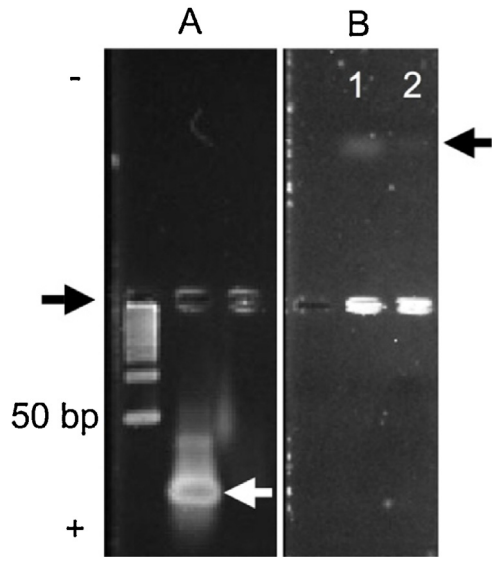

Fig. 1. Electrophoretic separation of chitosan nanoparticles containing 26 bp DNA and siRNA. Location of the positive and negative electrodes is indicated to the left of the gels. The location of the wells in which the samples were loaded is indicated by the black arrow on the left. A DNA size ladder is shown in the first lane of panel (A). The gel in panel (A) was stained with ethidium bromide following electrophoresis of DNA. The DNA (panel B, lane 1) and siRNA (panel B, lane 2) contained within the chitosan nanoparticles were intercalated with ethidium bromide prior to encapsulation and electrophoresis.

advanced drug delivery systems that may prove useful in the treatment of colon cancer.

According to the American National Cancer Institute, colon cancer is the third most common non-skin cancer. Liver metastases from colon cancer can be treated with chemotherapy and resection, but the five-year survival rate with liver resection is only 30–50%. This rate is even lower for patients undergoing chemotherapy alone (Kemeny, 2013). However, much of the poor prognosis when chemotherapy is employed can be attributed to the lack of target specificity and the ability of cancer cells to exhibit multidrug resistance (MDR) either intrinsically or acquired during treatment. The factors contributing to MDR include drug efflux, drug detoxification by enzymes, DNA damage repair, and reduced intracellular accumulation at the target site (Saraswathy & Gong, 2013). The latter is observed with many drugs designed to interact with DNA, where the drug is redistributed from nucleus to the cytoplasm. One promising approach that circumvents some of the issues associated with MDR is RNA interference (RNAi) technology. RNAi is a potent and highly specific gene-silencing phenomenon triggered by double-stranded RNA helices (Couzin, 2002; Deng et al., 2014). In this process, introduction of a 21–23 base pair siRNA into cells results in the degradation of homologous messenger RNA (mRNA), and ultimately decreased levels of the targeted protein. The success of RNAi depends on the effective knock-down of targets and efficient intracellular delivery of siRNA (Aagaard & Rossi, 2007).

Building on earlier research regarding chitosan as a carrier for delivering siRNA (Ganguly et al., 2014; Lu et al., 2010; Mao, Sun, & Kissel, 2010) specifically chitosan's buffering capacity and protonated amine groups, which neutralize the negatively charged siRNA, facilitating siRNA transport across the cell membrane and easy endosomal escape (Chang, Higuchi, Kawakami, Yamashita, & Hashida, 2010; Chaturvedi et al., 2011), studies using various cultured cell lines have shown high transfection efficiency for chitosan/plasmid DNA complexes. For example, positive transfection was observed in 30–50% of treated HEK293 cells (Strand, Issa, Christensen, Varum, & Artursson, 2008). Other studies concerning chitosan/siRNA nanoparticles were capable of reducing gene expression by about 50%, similar to the results obtained using the commercially available liposome-based transfection reagent, Lipofectamine (Garcia-Fuentes & Alonso, 2012; Yuan, Shah, Hein & Misra, 2010). Because β-catenin protein levels are elevated in 70–80% of colon cancer cells resulting in increased tumor cell proliferation (Wong & Pignatelli, 2002), a siRNA targeting mRNA coding for β-catenin may be a powerful therapeutic strategy to overcome some of the limitations associated with drugs that target DNA, which are often susceptible to drug efflux mechanisms and redistribution from nucleus to the cytoplasm (Saraswathy & Gong, 2013).

In an effort to develop a viable delivery system that can be adapted for *in vivo* applications, we report here the development of nanoparticles of chitosan and PEGylated chitosan for targeting siRNA toward β-catenin mRNA in human colon cancer cells. PEGylating chitosan is known to enhance colloidal stability for a longer circulation time, thus justifying its use. In this work, we compare both chitosan and PEGylated chitosan nanoparticles with Lipofectamine 2000 to determine whether these nanoparticles will mimic the success of a liposome-based approach in reducing β-catenin protein levels *in vitro*.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methoxy poly(ethylene glycol) succinimidyl valerate (mPEG-SVA) (MW of 5000) was purchased from Layson Bio (Arab, AL, USA). Lipofectamine 2000, β-catenin specific (ID# s436) silencer siRNA consisting of sequences where the sense strand is:

5′-GGACCUAUACUUACGAAAATT-3′, and the antisense strand is:

5′-UUUUCGUAAGUAUAGGUCCTC-3′ (13,300 Da) and the silencer select negative control siRNA #1, referred to below as scrambled RNA or "scrRNA" (13,400 Da) were obtained from Life Technologies (Carlsbad, CA, USA). A twenty-one base pair siRNA with an AlexaFluor 555 fluorescent tag on the 5′ end and a sense strand of 5′-UCUCCGAACGUGUCACGUTT-3′ and an antisense strand of 5′-ACGUGACACGUUCGGAGAATT-3′ was purchased from Qiagen (Germantown, MD, USA). The HCT-116 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. MALDI-TOF characterization of chitosan and mPEG-SVA

The molecular weights of chitosan and mPEG-SVA were determined from the matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) in a positive ion mode using a Bruker Daltonics Autoflex TOF/TOF mass spectrometer (Leipzig, Germany). A pulsed nitrogen laser with a wavelength of 337 nm was used to ionize the samples and Flexcontrol version 3.0 software was used to control the mass analyzer and to obtain the spectra. The matrix was prepared by dissolving 20 mg of 2,5-dihydroxybenzoic acid (Bruker Daltonics, Billerica, MA, USA) in 1 ml of 2:1 acetonitrile/0.1% trifluoroacetic acid (Sigma Aldrich, St. Louis, MO, USA). The mPEG-SVA and chitosan samples were dissolved in 5% acetic acid followed by dilution with matrix solution. The spectrum of chitosan was obtained in a linear mode between 50 and 250 kDa using the preset high mass method, while the spectrum of mPEG-SVA was obtained in a reflector mode between 2 and 10 kDa.

2.3. Synthesis of poly(ethylene glycol)-grafted-chitosan (PEGylated chitosan)

The mPEG-SVA was grafted onto chitosan using a carbodiimide-mediated reaction as previously described (Ravina et al., 2010). In