International Journal of Pharmaceutics 513 (2016) 431–437

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Cytotoxic and cytostatic side effects of chitosan nanoparticles as a non-viral gene carrier

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ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form 17 September 2016
Accepted 19 September 2016
Available online 19 September 2016

Keywords:
Gene delivery
Chitosan
Nanoparticles
pDNA
Cytotoxicity
Monocytes
Cancer cells

ABSTRACT

Although chitosan nanoparticles (CNs) became a promising tool for several biological and medical applications owing to their inherent biocompatibility and biodegradability features, studies regarding their effects on cytotoxic and cytostatic properties still remain insufficient. Therefore, in the present study, we decided to perform comprehensive analysis of the interactions between CNs–pKindling-Red-Mito (pDNA) and different cell line models derived from blood system and human solid tissues cancers. The resulting CNs-pDNA was investigated in terms of their cellular uptake, transfection efficiency, and physico-chemical, cytotoxic and cytostatic properties. The nanoparticles showed high encapsulation efficiency and physical stability for various formulations even after two days time period. Moreover, high gene expression levels were observed after 96 h of transfection. CNs-pDNA treatment, despite the absence of oxidative stress induction, caused cell cycle arrest in G0/G1 phase and as a consequence led to premature senescence which turned out to be both p21-dependent and p21-independent. Also, observed DNMT2 upregulation may suggest the activation of different pathways protecting from the results of CNs-mediated stress. In conclusion, treatment of different cell lines with CNs-pDNA showed that their biocompatibility was limited and the effects were cell type-dependent.

1. Introduction

Gene therapy is one of the most promising strategies for treatment of various diseases such as cancer and inflammatory, neurological, cardiovascular or metabolic disorders (Ibraheem et al., 2014; Ragelle et al., 2014). Simultaneously, the choice of a good transfection agent for delivery of nucleic acids is critical for each gene transfer procedure, however most commercially available transfection agents have still some limitations (Perez-Martinez et al., 2011). Recently, in gene delivery, viral vectors have been used due to the high transfection efficiency compared to non-viral gene delivery systems. However, toxicity issues such as immunogenicity and mutagenicity limit the clinical applications of the viral vectors. Taking into account safety considerations, non-viral vectors are thought to be more promising nucleic acid carriers compared to viral vectors (Ishii et al., 2001).

Among various non-viral vectors, chitosan (CS) is one of the most commonly studied polymer since its positive charges under slightly acidic conditions allow its interaction with nucleic acids and thus formation of complexes of nanoparticles (Ragelle et al., 2014). On the other side, it is known that many biological effects caused by nanoparticles depend on their size, shape, used dose, behavior of nanoparticles in enviroment (*i.e.*, whether or not their aggregation occurs) and cell type (*i.e.*, suspended versus adherent cell types or healthy versus cancer cell types) (Fu et al., 2014; Mytych et al., 2015a, 2015b). It has been shown that plasmid DNA loaded chitosan nanoparticles (CNs-pDNA) did not cause any cytotoxicity to macrophages (which differentiate from monocytes) (Chellat et al., 2005). Despite these promising results, potential side effects including cytostatic or cytotoxic effects caused by biodegradable nanomaterials like CNs or/and type of used expression vector system on different cell types are poorly

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http://dx.doi.org/10.1016/j.ijpharm.2016.09.058
0378-5173/

understood. Previously, some researchers have reported that GFP transduction might affect the cytophysiology of the targeted cell. For example, it is widely accepted that tagged chromosome proteins may affect the motion of chromosomes. In general, green fluorescent protein (GFP) is considered nontoxic both in *in vitro* and *in vivo* systems. However, there are some reports showing side effects of GFP in *in vitro* and *in vivo* studies, but the details of molecular mechanism of these interactions still remain unclear (Agbulut et al., 2006, 2007; Baens et al., 2006; Huang et al., 2000; Liu et al., 1999). Recently, it has been shown that Ku80 attenuates cytotoxicity induced by green fluorescent protein transduction independently of non-homologous end joining (Koike et al., 2013).

Moreover, the application of biodegradable nanomaterials as nucleic acid delivery systems for living organisms including humans raises also other important questions about the impact of CNs on efficiency of pDNA condensation. Matsumoto et al. (2009) showed the differences in the transcriptional activity of pDNAs transfected into cells using either cationic lipids or cationic polymers (Matsumoto et al., 2009).

Therefore, in this study, we aimed to use the ionic gelation method for the encapsulation of pKindling-Red-Mito vector into chitosan nanoparticles and to evaluate their potential as safe-nanocarriers on different cell line models derived from human solid tissues cancers (HeLa and MDA-MB-231) and monocytes (derived from peripheral blood cancer—THP-1).

2. Materials and methods

2.1. Materials

Plasmid DNA (pKindling-Red-Mito) encoding red fluorescent protein (KFP) and neomycin resistance gene were purchased from Evrogen JSC, Russia. All other reagents, if not otherwise mentioned, were purchased from Sigma Aldrich, Poland and were of analytical grade.

2.2. Preparation and characterization of CNs-pDNA

CNs-pDNA were synthesized following the Calvo's ionic gelation procedure (Calvo et al., 1997) with some modifications. According to this procedure, low molecular weight chitosan (50–190 kDa and the degree of acetylation of 75–85%) was used with tripolyphosphate (TPP) as a crosslinker. Chitosan stock solution (0.4%) was prepared by dissolving the chitosan powder in 1% (v/v) acetic acid under magnetic stirring. The solution was filtered through sterile 0.45 μm membrane and the pH was adjusted to 5.5. Nanoparticles were spontaneously obtained upon addition of TPP (0.1%) and pDNA in nuclease free water to chitosan solution (chitosan to TPP weight ratio of 4:1). CNs-pDNA at weight ratios of 500:1, 300:1 and 100:1 were generated at room temperature by adding a constant pDNA concentration of 10 μg/ml. After complex formation, CNs-pDNA solution was centrifuged at 13500 rpm for 30 min and then the pellet was dissolved in 2 mL of nuclease free water (pH 7.4) for further use in the *in vitro* studies.

CNs-pDNA were characterized using dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments Ltd, UK) for determination of mean particle diameter (Z-Average), polydispersion index (P.I.) and zeta potential values. Each measurement was performed in triplicate at a temperature of 25 °C with an angle of 173° in ultrapure water.

For the morphological characterization of particles, atomic force microscopy (AFM) was used. All the AFM samples have been deposited on freshly cleaved muscovite mica, incubated for 10 min, and then rinsed with ultrapure water, gently flushed with a stream of nitrogen for drying. The Height Sensor and Peak Force Error AFM images were analyzed with Nanoscope Analysis software (v. 1.40 Bruker Corporation, Germany). Mean diameter and zeta potential of the nanoparticles were estimated by the software provided by Malvern Instruments Ltd.

2.3. Evaluation of encapsulation efficiency

To determine the amount of plasmid DNA encapsulated in the chitosan nanoparticles, the difference between the total amount of pDNA and the amount of non-entrapped pDNA remaining in the supernatant after centrifugation (13,500 rpm, 30 min) was calculated. For this purpose, the supernatant was used with the corresponding of blank formulation for the calibration curve. It was spectrophotometrically analyzed at 260 nm for pDNA concentration by NanoDrop™ 2000/2000c Spectrophotometers.

Furthermore, the physical stability of CNs-pDNA nanoparticles in physiological environment was also determined by 1% agarose gel electrophoresis in Tris-borate EDTA buffer (4.45 mM Tris-base, 1 mM sodium EDTA, 4.45 mM boric acid) at 120 V for 30 min. Each formulation was loaded on the gel after it was incubated in DMEM medium at 37 °C for 24 h and 48 h.

2.4. Cell culture

Human cervical (HeLa; ATCC), breast (MDA-MB-231; ATCC) (seeding density 3×10^3 cells/cm^2) and peripheral blood (THP-1; ECACC) (seeding density 2×10^5 cells/ml) cancer cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (for HeLa and MDA-MB-231) or Roswell Park Memorial Institute medium (RPMI 1640) (for THP-1). They were supplemented with 10% fetal calf serum (FCS) and an antibiotic and antimycotic mixed solution (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B) at 37 °C in a humidified atmosphere in the presence of 5% CO_2 until they reached confluence. Typically, cells were passaged by trypsinization (adherent cells) or direct dilution (suspension cells) to a seeding density.

2.5. In vitro *transfection studies*

Cells were seeded onto 96-well plate at a density of 3×10^3 (HeLa and MDA-MB-231) or 2×10^4 (THP-1) cells per well and treated with CNs-pDNA at different concentrations and weight ratios of 500:1, 300:1 and 100:1. After 24 h incubation, the medium and complexes were removed, media was replaced with fresh medium additionally containing neomycin sulphate (1 mg/ml) as selection agent. The live cells were examined after 96 h with In Cell Analyzer 2000 (GE Healthcare, UK) equipped with a high performance CCD camera. The percentage of transfected cells (%) were calculated by qualitative analysis of the acquired images. Each transfection experiment was carried out as triplicate. Stable cell lines (stable CNs-pDNA) were obtained by continuous long-term culture in medium supplemented 1 mg/ml neomycin sulphate.

2.6. Cell viability and cell cycle analysis

MTT assay was performed to evaluate the cell viability after 24 h treatment with different formulation of CNs-pDNA (500:1, 300:1 and 100:1). Briefly; cells were seeded onto 96-well plates at a density of 3×10^3 (HeLa and MDA-MB-231) or 2×10^4 (THP-1) cells per well. After 24 h treatment, the formulations were discarded and replaced with MTT-containing medium (500 μg/ml). After 4 h incubation at 37 °C, formazan crystals were dissolved in anhydrous dimethyl sulfoxide (DMSO). Absorbance was read at 570 nm (measurement wavelength) and at 630 nm (reference wavelength) by Tecan Infinite® M200 absorbance mode microplate reader.