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Gene regulation with carbon-based siRNA conjugates for cancer therapy

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ABSTRACT

We report fluorescent carbon nanoparticle (FCN)-based small interfering RNA (siRNA) conjugates (C-siRNA) for gene regulation and cancer therapy. The C-siRNA has a core of chitosan-derived FCN and a shell of siRNA, and can down-regulate the expression of polo-like kinase-1 (Plk1), a master regulator of mitosis, via siRNA targeting Plk1 (siPlk1), for cancer therapy. The required amount of the FCNs is only ~1/30 of that of the gold nanoparticles in delivering equal amount of siRNA. The C-siPlk1 led to ~80% knockdown of cellular Plk1 mRNA in A375 cells, and induced apoptosis of the A375 cells (31.9%) and MCF-7 cells (20.33%), much higher than those by commercial nonviral gene delivery vectors, such as Lipofectamine 2000 in both cell lines (apoptosis rate < 10%). After the C-siPlk1 was administrated to A375 tumor-bearing mice intravenously, the tumor volume was less than 1/11 of the control groups. The C-siRNA can thus be powerful tools for gene delivery and gene therapy.

1. Introduction

Gene therapy has emerged as innovative and powerful means for the treatment of challenging diseases such as cancer [1,2]. Non-viral delivery of genes is considered the safest amongst the choices in delivery of genes [3,4]. However, the delivery of synthetic nucleic acids is still a major challenge for gene regulation and therapy [5]. At present, none of the vectors, such as cationic polymers, liposomes, and modified viruses is ideal for systemic delivery due to various shortcomings such as inability to be degraded naturally, toxicity, and severe immunogenicity [6–8].

Nanoparticles (NPs)-oligonucleotide conjugates, in which NPs are coated with oligonucleotides, have become useful for drug delivery, gene therapy, and diagnostics [9–12]. In contrast with the traditional means of delivering nucleic acids, these NPs-oligonucleotide structures show high level of cellular uptake and transfection efficiency without resorting to any other transfection agents [13]. Moreover, the structure showed resistance against degradation by nuclease, minimal immune response, low toxicity, and highly effective gene regulating capabilities [14–16]. NPs-oligonucleotide may be promising systems for gene delivery and gene therapy [17].

Many existing NPs-oligonucleotide structures are composed of gold nanoparticle (AuNPs) cores and oligonucleotide shells (AuNPs-oligonucleotides) [17]. However, the application of AuNPs is limited by several factors: i) their long term toxicity is not completely characterized [18]; ii) they usually show high scattering background from the cells by dark field imaging [19], limiting their applications for cellular imaging. Multifunctional materials are needed for broadening the application of NPs-oligonucleotide not merely on gold-based conjugates. Carbon-based materials are attractive in biomedicine because they have tunable fluorescence [20], high loading efficiency [21], and convenient preparation [22]. Fluorescent carbon nanoparticles (FCNs), such as fullerene, single-walled carbon nanotubes, graphene, nanodiamonds are promising agents for diagnostic and therapeutic applications because they have high photostability (their fluorescence does not change even after continuous photobleach) [23], low toxicity [24–26], as well as good biocompatibility [27,28]. In addition, FCNs could be efficiently and rapidly excreted from the body *in vivo* [29]. FCNs thus show great potential in biomedical research. However, the materials mentioned above always involve tough synthetic processes, expensive original materials, great energy-consuming devices, and low yield. Recently, carbon dots have attracted increasing attention and have been utilized as imaging-guided nanocarriers for the delivery of chemotherapeutics and genes [30–32]. These carbon dots need post-modifications with active groups through

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tedious works. Besides, highly positive charges will be formed by the modification with cationic polymers, which is usually cytotoxic and disadvantageous in the biomedical application.

More recently, researchers have prepared highly amino-functionalized FCNs in gram-scale through one-step, inexpensive, and convenient process by hydrothermal reaction of chitosan at a mild temperature (180 °C, instead of the typical 300 °C required) [23]. In this process, the chitosan was carbonized to nanoscale particles by hydrothermal reaction. The nitrogen doping derived from chitosan can make the surface sites more stable to facilitate surface-confined electrons and holes which may result in the fluorescence [33–35]. Combined with green synthesis, ease in labeling, and other favorable properties, it is reasonable to assume that the chitosan-derived FCNs provide promise for large-scale preparation and broad applications in theranostics. Chitosan-derived and amino-functionalized FCNs previously have not been utilized to load and transport nucleic acids, especially siRNA.

Herein, we report fluorescent, chitosan-derived carbon nanoparticles-based siRNA conjugates (C-siRNA) and their applications in gene regulation for cancer therapy. We functionalized the FCNs with thiolated small interfering RNA (siRNA) targeting *Plk1* gene (siPlk1) to form C-siPlk1. *Plk1* is a master regulator of mitosis, whose overexpression is often observed in tumor cells, such as A375 (human melanoma) and MCF-7 (human breast cancer) cells. The inhibition of *Plk1* is considered to be an effective way to treat tumors [36]. Previous report has indicated that the inhibition of *Plk1* by siRNA can induce tumor cell apoptosis and suppress tumor progression [37]. We suppose that the cancer therapy will be more effective by appropriate siPlk1 delivery system. In this study, the siPlk1 was delivered in the form of C-siPlk1 and we evaluated if the C-siPlk1 could be internalized by tumor cells without help of any transfection agent and knock down target gene (*Plk1*) both *in vitro* and *in vivo* to inhibit cancer progression.

2. Materials and methods

2.1. Synthesis of fluorescent carbon nanoparticles (FCNs)

In a typical procedure, amino-functionalized FCNs were synthesized as previous report with slight modifications [23]. Briefly, low molecular weight chitosan (Sigma-Aldrich, USA) was dispersed in distilled water with a concentration of 5 mg/mL, sealed into a Teflon-equipped stainless steel autoclave, and placed in an oven followed by hydrothermal treatment at 180 °C for 12 h. After the reaction, the autoclave was cooled down naturally and the solution was centrifuged (14 000 g) for 15 min and the supernatant was purified by ultra-filtration to obtain the FCNs.

2.2. Cell culture

A375 and MCF-7 cell lines were obtained from Institute of Basic Medical Sciences (Beijing, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin in 5% CO_2 at 37 °C.

2.3. Cytotoxicity assay

The A375 or MCF-7 cells were seeded into 96-well plates with a density of 5×10^3 cells per well (Corning, USA) in the DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin for 24 h prior to the experiment. The culture medium was replaced with 200 μL FCNs solutions with varied concentrations (10–350 μg/mL). Each concentration was replicated in 5 wells. The cells were incubated for another 24 h before the medium in each well was replaced with freshly prepared serum-free medium with 10% CCK-8 (Dojindo, Japan) and the cells were incubated for another 2 h. The optical density readings were performed using a multimode plate reader (EnSpire, PerkinElmer, USA) at a wavelength of 450 nm. The absorbance was read relative to the blank well. Cell viability (%) in each well was calculated by OD_{450} test/OD_{450} control × 100%.

2.4. Preparation of C-siPlk1 conjugates

To prepare the C-siPlk1 conjugates, all components should be free of nucleases, such as DNase or RNase, which degrades siRNA ligands. To maximize the coverage of the siRNA on the FCN shell surface, the synthetic route was referred to the previous report [38]. The heterobifunctional cross-linker *N*-[γ-maleimidobutyryloxy] sulfosuccinimide ester (Sulfo-GMBS) was used since it is a heterobifunctional cross-linker with amine-reactive *N*-hydroxysuccinimide (NHS) ester and maleimide groups, which allow covalent conjugation of amine- and thiol-containing molecules effectively. The amine-rich FCNs were activated with amine-reactive Sulfo-GMBS to introduce thiol-reactive maleimide groups. The thiolated siPlk1 were cleaved with dithiolthreitol (DTT) (0.1 M, 2 h) and purified on size exclusion columns (Sephadex™ G-25 DNA Grade, GE Healthcare). After the addition of thiolated siPlk1, 3 M NaCl was added to a final concentration of 0.3 M over 4 h, which increased the coverage of siRNA on the nanoparticle surface. The final mixture was shaken for ~24 h to complete the siRNA functionalization process. The particles were ultra-filtrated repeatedly to remove unbound siRNA and impurities. Next, the resulting product was resuspended in sterile phosphate buffered saline (PBS) for cell studies. The siRNAs used in the experiments were listed below:

siRNA duplex designed against Plk1 mRNA:
Sense strand: 5′-UGAAGAAGAUCACCCUCCUUAdTdT-Thiol-3′;
Sense strand: 5′-Cy5-UGAAGAAGAUCACCCUCCUUAdTdT-Thiol-3′;
Antisense strand: 5′-UAAGGAGGGUGAUCUUCUUCAdTdT-3′ [39].
Scrambled siRNA:
Sense strand: 5′-UUCUCCGAACGUGUCACGUdTdT-Thiol-3′;
Antisense strand: 5′-ACGUGACACGUUCGGAGAAdTdT-3′.

2.5. Fourier transform infrared (FT-IR) spectrum characterization

Chitosan and its derivatives were mixed with KBr and compressed into disks, and their FT-IR spectra were recorded by an FT-IR spectrometer (Spectrum 2000, Perkin Elmer).

2.6. High resolution transmission electron microscopy (HR-TEM) characterization

The FCNs or C-siPlk1 were suspended in water and added to carbon-coated 200-mesh copper grids and air-dried at room temperature before observing under TEM with a field emission electron source (Tecnai G2 F20 U-TWIN, FEI). The statistical analysis was carried out using Nano measurer 1.2 software.

2.7. Loading capability evaluation

To determine the loading efficiency of the FCNs, C-Cy5 siPlk1 was prepared as C-siPlk1 conjugations. Next, each set of samples using varying concentrations of FCNs or C-Cy5 siPlk1 was prepared. By using a multimode plate reader (EnSpire, PerkinElmer, USA), we can establish calibration curves for analysis of FCNs or Cy5 siPlk1 (FCNs excitation, 360 nm; Cy5 siPlk1 excitation, 650 nm). The fluorescence of the experimental samples was collected through