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Original Research

The cellular uptake of antisense oligonucleotid of E6 mRNA into cervical cancer cells by DOPE-modified hydroxyapatite nanoparticles

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Abstract

Objective(s): Although several chemical and physical methods for gene delivery have been introduced, their cytotoxicity, non-specific immune responses and the lack of biodegradability remain the main issues. In this study, hydroxyapatite nanoparticles (NPs) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)-modified hydroxyapatite NPs was coated with antisense oligonucleotide of E6 mRNA, and their uptakes into the cervical cancer cell line were evaluated.

Materials and Methods: Calcium nitrate and diammonium phosphate were used for the synthesis of the hydroxyapatite nanoparticle. Thus, they were coated with polyethylene glycol (PEG), DOPE and antisense oligonucleotide of E6 mRNA using a cross-linker. Then, hydroxyapatite NPs and DOPE-modified hydroxyapatite NPs were incubated 48 hours with cervical cancer cells and their uptakes were evaluated by fluorescent microscopy.

Results: The hydroxyapatite NPs had different shapes and some agglomeration with average size of 100 nm. The results showed DOPE-modified hydroxyapatite NPs had higher uptake than hydroxyapatite NPs ($P<0.05$).

Conclusions: Hydroxyapatite NPs conjugated with DOPE are a good choice for gene delivery and silencing of viral genes in cervical cancer cells, but their efficacy should be addressed more in future studies.

Keyword(s): Cervical cancer cells, DOPE, Hydroxyapatite nanoparticles, Oligonucleotide

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Introduction

Nanoparticles (NPs) are solid colloidal materials, ranging from 10 to 1000 nm (1). NPs have sites in which the biologically active substances are entrapped, encapsulated, or adsorbed onto their surface. On the other hand, NPs were described as nanopellets and nanocapsules, adjuvants, drug delivery systems, and etc (2). Cationic polymer including polybutyl-cyanoacrylate (PBCA), poly-isohexyl-cyanoacrylate (PIHCA), and poly-hexylcyanoacrylate (PHCA) were first introduced as gene delivery systems for plasmid DNA by Bertling et al. in 1991, who compared their efficiency with other delivery systems (3). Based on previous studies, oligodeoxynucleotides (ODNs) can be bound to NPs (4-6).

Cationic polymer can be adsorbed on the inorganic material such as hydroxyapatite which is chemically similar to the mineral component of bones and hard tissues in mammals (7, 8). It is one of few materials that are classified as bioactive, meaning that it would support bone growth and osseointegration when used in orthopaedic, dental and maxillofacial applications (9). The chemical nature of hydroxyapatite lends itself to substitution, meaning that it is not uncommon for non-stoichiometric hydroxyapatites to exist. Advanced implants, e.g. hip replacements, dental implants and bone conduction implants, are coated with hydroxyapatite which may promote osseointegration (9). Porous hydroxyapatite can be used for local drug delivery and for repair early lesions in tooth enamel (10). On the other hand, hydroxyapatite can be employed for gene delivery same as other gene carrier (11). To improve characteristics of this material, some polymeric substance may be added such as hexadecyltrimethylammonium bromide (CTAB) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (12). These are hydrophobic cationic detergents that were used in combination with hydrophobic polymer particles and other NPs as adsorption enhancers for hydrophilic anionic oligonucleotides (13). In this study, we synthesized hydroxy-apatite NPs coated with PEG, modified by DOPE and covered by antisense oligonucleotide of E6 mRNA. Then, their uptake into cervical cancer cells was investigated.

Materials and Methods

Materials

Calcium nitrate, ammonium phosphate, sodium hydroxide, carbonic acid, Giemsa and methanol were purchased from Merck (Germany). The carboxylated polyethylene glycol (PEG), 1-Ethyl-3-[3-dimethy-laminopropyl] carbodiimide hydrochloride (EDC), DOPE, and RPMI 1640 medium were obtained from Sigma-Aldrich, USA. Antisense oligonucleotid labeled with HEX was purchased from Takapo Zyst Co.

Synthesis of hydroxyapatite NPs and their modification

Calcium nitrate (36.15 g) was dissolved in deionized water (525 mL). Di-ammonium phosphate (12 g) was dissolved in deionized water 375 ,L). Then, a portion of each solution (25 mL) was added and its pH was adjusted to pH = 11. The mixture was incubated 6 h at room temperature for growth process. After incubation, NPs were washed three times with deionized water and centrifuged at 5000 rpm for 5 min. Then, carboxy-PEG (1 mL) was added to washed NPs (1 g) and incubated 30 min at 37 °C. After incubation, NPs coated with carboxy-PEG were centr-ifuged. Then, 1 mL of DOPE and 1 mL of EDC was added to NPs coated with carboxy-PEG (1 g), incubated for 30 min at 37 °C and centrifuged at 5000 rpm for 5 min. In the next step, antisense oligonucleotid (1 mL, 1000 nM) of E6 mRNA was added to DOPE-coated NPs and incubated for 1

h at 37 °C and washed by deionized water and centrifuged at 5000 rpm for 5 min. For control, washed hydroxyapatite NPs were directly exposed to antisense oligonucle-otid (1 mL, 1000 nM) of E6 mRNA and incubated for 1 h at 37 °C, washed, and centrifuged at 5000 rpm for 5 min.

Characterization of NPs

For characterization, three methods were used including Scanning Electron Microscope (SEM) for morphology, Dynamic Light Scattering (DLS) for size distribution, and Fourier transform infrared spectroscopy (FTIR) for surface characteristics.

Uptake of NP

100 µL of serial concentrations (500, 250, 125, 62 and 31 µg/ml) of hydroxyapatite NPs and final modified hydroxyapatite NPs were separately added to 100 µL of cervical cancer cells (10^3 cells) which suspended in the RPMI1640 with 10% fetal calf serum, and incubated for 48 hours at 37 °C. Then, the cells were washed twice with normal saline. Then, 100 µL of carbonic acid (1 mL, carbonic acid) was added and the cells were incubated for 24 h. After incubation, the optical absorbance (OA) of each well was read by FTIR at 2999 cm^{-1} and the level f uptake was measured separately for the cells exposed to hydroxyapatite NPs and modified hydroxyapatite NPs. Finally, the fluorescent of cells which incubated with labeled hydroxyapatite NPs and modified hydroxyapatite NPs was studied. Thus, cells were fixed with methanol and examined by fluorescent microscope with magnification 1000.

Results

Characterization of NPs

The SEM image of modified hydroxyapatite NPs is shown in the Figure 1. As seen, they exhibited different forms and a high degree of agglomeration was seen. The size distribution of modified hydroxyapatite NPs is shown in the Figure 2. The mean, the standard deviation and the polydispersity index (PDI) were 100 nm, 38 nm and 0.15, respectively. The FTIR spectrum (Figure 3) confirmed the attachment of DOPE and oligonucleotide to the hydroxyapatite NPs.

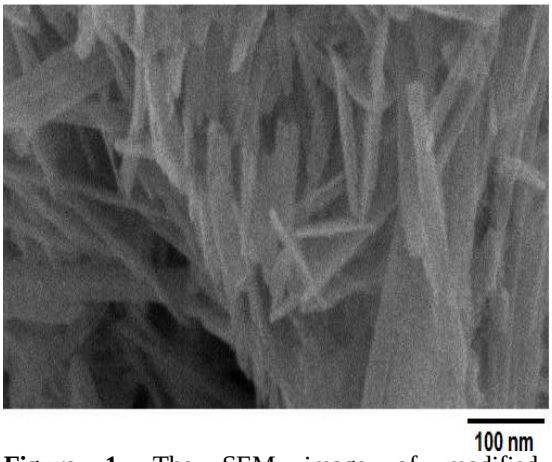

Figure 1. The SEM image of modified hydroxyapatite NPs.

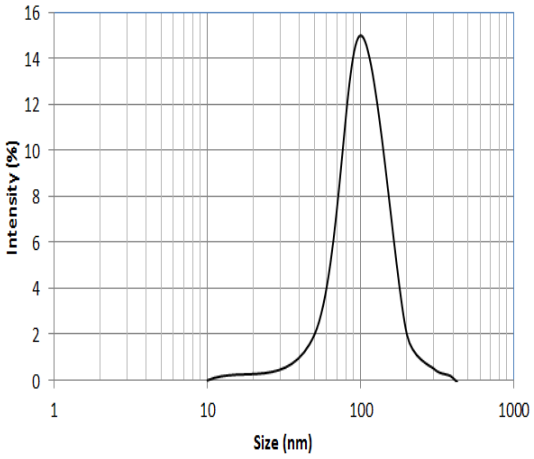

Figure 2. The size distribution of modified hydroxyapatite NPs.

Uptake of NP

Figure 4 shows the uptake of oligonucleotide by both hydroxyapatite NPs and modified hydroxyapatite NPs. As shown, modified hydroxyapatite NPs had higher uptake than hydroxyapatite NPs. In both, the pattern of uptake was dose dependent. The microscopic pictures of cancer cells which treated with hydroxyapatite NPs and modified

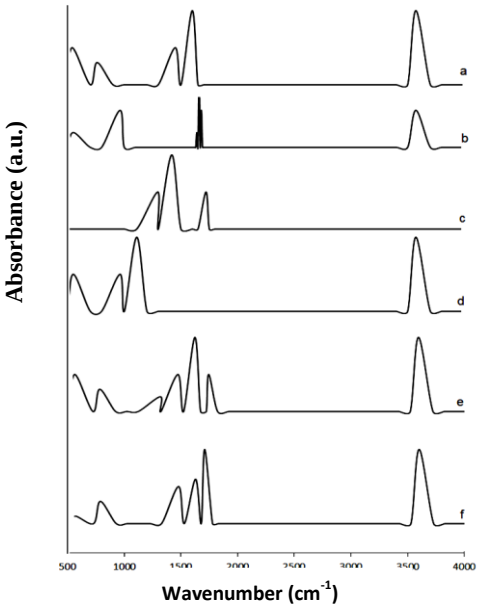

Figure 3. The FTIR spectrum of oligonucleotide (a), DOPE (b), PEG (c), hydroxyapatite NPs (d), hydroxyapatite NPs covered by oligonucleotide (e), modified hydroxyapatite NPs covered by oligonucleotide (f).

hydroxyapatite NPs are shown in the Figure 5a and Figure 5b, respectively. Remarkably, the cancer cells which treated with hydroxyapatite NPs had less florescent than cells treated with modified hydroxyapatite NPs.

Discussion

One of the challenges of the in vivo application of gene therapies is the efficient delivery, especially across the plasma membrane to the cytoplasm of target cells (14).

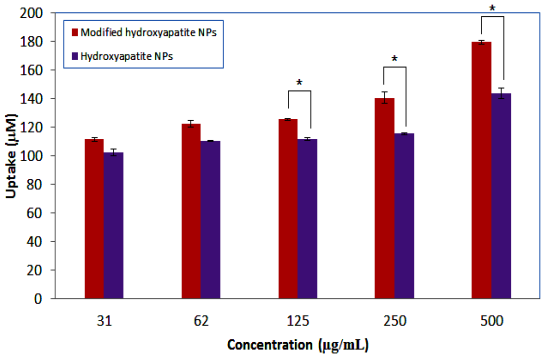

Figure 4. The uptake of oligonucleotide by hydroxyapatite NPs and modified hydroxyapatite NPs. * $p<0.05$ compared with modified hydroxyapatite NPs.

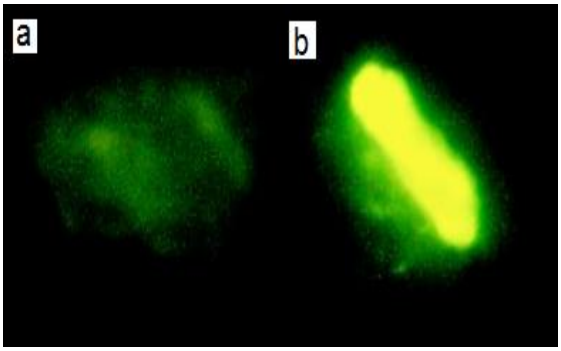

Figure 5. The microscopic pictures of cancer cells which treated with hydroxyapatite NPs (a) and modified hydroxyapatite NPs (b).

Nanomedicine is a newly emerging practice that fuses nanotechnology and medicine. NPs have been used as diagnostic probes in targeted therapy (15). Hydroxyapatite is the principal inorganic constituent of human bones and teeth and its molecular formula is $Ca_{10}(PO_4)_6(OH)_2$. Synthetic HAP crystals are now widely used in medical implants and as coatings on prostheses (7-9). Compared to viral vectors which possess risks of pathogenicity and immunogenicity, synthetic HAP NPs have favorable biocompatibility and high osteoconductivity and/or osteoinductivity without immunogenicity or pro-inflammatory features (16).

HAP not only can directly inhibit the proliferation of cancer cells but also it can be used as a gene delivery system due to its safety, economy and efficiency (11, 17).

Moreover, hydroxyapatite can be modified in order to increase its gene delivery capability (18, 19). DOPE is a phospholipid molecule which has a positive charge and can be employed for inducing positive charge on the NPs (20). On the other hand, DOPE helps oligonucleotide to escape from lysosome (21). The endolysosomal escape of gene carriers is crucial in enhancing the efficacy of their macromolecular payload, esp-ecially the payloads that are susceptible to lysosomal degradation (22). DOPE that enable the endo-lysosomal escape of macromolecules

such as DNA are inte-rested by its ability to carry various classes of genes and therapeutic agents (23). This study showed that the uptake of oligonucleotide by DOPE-modified hydroxy-apatite NPs washigher than that of hydroxyapatite NPs alone. Both showed a dose-dependent pattern of uptake which is common in gene delivery. The microscopic pictures of cancer cells showed that cells which treated with DOPE-modified hydroxyapatite NPs had higher florescent intensity than hydroxyapatite NPs alone.

Conclusions

Hydroxyapatite NPs conjugated with DOPE are a good choice for gene delivery aimed at silencing of genes in cervical cancer cells and could be a suitable carrier for the purposes of gene therapy. Their efficacy should be addressed more in future studies.

Acknowledgments

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