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Original article

CpG oligodeoxynucleotide-loaded PAMAM dendrimer-coated magnetic nanoparticles promote apoptosis in breast cancer cells

Negar Taghavi Pourianazar[a,*], Ufuk Gunduz[a,b,*]

[a] Middle East Technical University, Department of Biotechnology, 06800 Ankara, Turkey
[b] Middle East Technical University, Department of Biological Sciences, 06800 Ankara, Turkey

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ABSTRACT

One major application of nanotechnology in cancer treatment involves designing nanoparticles to deliver drugs, oligonucleotides, and genes to cancer cells. Nanoparticles should be engineered so that they could target and destroy tumor cells with minimal damage to healthy tissues. This research aims to develop an appropriate and efficient nanocarrier, having the ability of interacting with and delivering CpG-oligodeoxynucleotides (CpG-ODNs) to tumor cells. CpG-ODNs activate Toll-like receptor 9 (TLR9), which can generate a signal cascade for cell death. In our study, we utilized three-layer magnetic nanoparticles composed of a Fe_3O_4 magnetic core, an aminosilane (APTS) interlayer and a cationic poly(amidoamine) (PAMAM) dendrimer. This will be a novel targeted delivery system to enhance the accumulation of CpG-ODN molecules in tumor cells. The validation of CpG-ODN binding to DcMNPs was performed using agarose gel electrophoresis, UV-spectrophotometer, XPS analyses. Cytotoxicity of conjugates was assessed in MDA-MB231 and SKBR3 cancer cells based on cell viability by XTT assay and flow cytometric analysis. Our results indicated that the synthesized DcMNPs having high positive charges on their surface could attach to CpG-ODN molecules *via* electrostatic means. These nanoparticles with the average sizes of 40 ± 10 nm bind to CpG-ODN molecules efficiently and induce cell death in MDA-MB231 and SKBR3 tumor cells and could be considered a suitable targeted delivery system for CpG-ODN in biomedical applications. The magnetic core of these nanoparticles represents a promising option for selective drug targeting as they can be concentrated and held in position by means of an external magnetic field.

1. Introduction

CpG-oligodeoxynucleotides (CpG-ODNs) can stimulate the immune system *via* interaction with Toll-like receptor 9 (TLR9) [1,2]. These unmethylated CpG-ODN motifs are characteristic for bacterial and viral DNA. In humans, TLR9 is expressed in numerous cells of the immune system and also in cancer cells, including breast [3,4], brain [5,6], gastric [7,8], lung [9,10], and prostate [11,12]. TLR9, specifically recognizing unmethylated CpG oligonucleotides in vertebrates, is localized at endoplasmic reticulum. Then, it is translocated to the endosomal/lysosomal compartment for ligand recognition [13,14]. After binding to ligand, TLR9 and its associated adaptors, such as MyD88 and TRIF, recruit intracellular signaling mediators that activate transcription factors, such as nuclear factor κB (NF-κB) [15,16]. Activation of TLR9 leads to triggering apoptosis in various types of malignant cells including breast cancer cells. Therefore, TLR9 agonist could be considered as candidates for cancer treatment [17–21].

However, successful transfer of the CpG-ODN to the tumor site is dependent on the development of an efficient delivery vector to overcome various hurdles, such as rapid degradation by serum nucleases and poor diffusion across the cell membrane. Due to their growing clinical significance, different formulations and delivery systems have been designed to improve the stability and efficiency of cellular uptake of CpG-ODNs [22,23].

Magnetic nanoparticles (MNPs) based on iron oxide as targeted drug delivery system have received specific attention due to their simplicity, ease of synthesis, and ability to tailor their properties for special biological purposes. These nanoparticles carrying the anticancer agent can be targeted to the tumor site, and accumulated in cancer cells by the help of an implanted permanent magnet or an externally applied field (Fig. 1). However, the use of magnetic nanoparticles needs lot of surface modification so as to protect them from reticuloendothelial system and to increase their

* Corresponding authors at: Department of Biological Sciences, Middle East Technical University, Ankara, 06800, Turkey. Fax: +90 312 210 7976.
E-mail addresses: Negar_taghavi22@yahoo.com (N. Taghavi Pourianazar), ufukg@metu.edu.tr (U. Gunduz).

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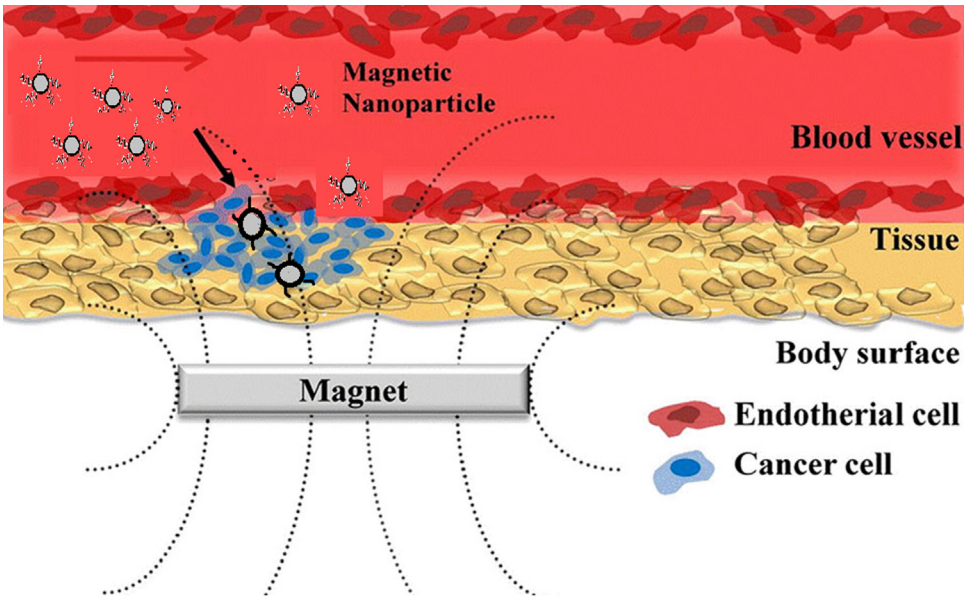

Fig. 1. Schematic representation of magnetic drug delivery system on application of an external magnetic field.

stability *in vivo*. Organic ligands such as poly(amidoamine) (PAMAM) dendrimer, polyethylene glycol, dextran, and aminosilanes are commonly used to stabilize these nanoparticles [23,24].

PAMAM dendrimers are a class of spherical, well-designed branching polymers with abundant terminal groups on the surface which can form stable complexes with plasmid DNA and oligonucleotides. Various useful characteristics of PAMAM dendrimers such as their nanometer size and manageable molecular weight, their biocompatibility, and non-immunogenicity make them suitable options as nanovectors with improved stability and bioavailability [22,25–30].

In this study, the advantages of MNPs and PAMAM dendrimers were fully used to solve the problems associated with CpG-ODN delivery, such as poor diffusion across cell membrane and rapid degradation by exonuclease or endonuclease, by fabricating nanoscale delivery system composed of MNPs covered with different generations of PAMAM dendrimers. The synthesized dendrimer-coated magnetic nanoparticles (DcMNP) were used to complex with CpG-ODN (Fig. 2). Then, characterization of the CpG-ODN nanoparticles was performed. Our results showed that the surface dendritic structure of the DcMNPs allows the efficient attachment of CpG-ODN to the surface of DcMNPs and markedly enhanced its delivery. Finally, the cellular internalization and cytotoxicity effect of these complexes was evaluated in MDA-MB231 and SKBR3 breast cancer cell lines.

2. Material and methods

2.1. Materials

Ferric chloride hexa-hydrate ($FeCl_3{\cdot}6H_2O$), ferrous chloride tetra-hydrate ($FeCl_2{\cdot}4H_2O$), ammonia solution (NH_3) (32%), aminopropyltriethoxysilane (Aminosilane or APTS) (NH_2 $(CH_2)_3$–Si–$(OCH_3)_3$), methylacrylate, methanol, ethanol, ethylenediamine, PBS (phosphate-buffered saline) were purchased from Sigma–Aldrich and acetic acid was purchased from Merck. Type B CpG-ODN 2006was purchased from InvivoGen. The sequence of CpG-ODN 2006 was 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′. Annexin-V-Fluos staining kit was purchased from Roche Life Science.

2.2. Synthesis of PAMAM dendrimer-coated magnetic nanoparticles (DcMNPs)

For the synthesis of MNP core (Fe_3O_4), the co-precipitation method was used. The main procedure starts with the co-precipitation of Fe^{2+} and Fe^{3+} ions (1:2 molar ratios) with addition of ammonia solution by passing nitrogen gas, and vigorous stirring at 90 °C. The precipitated magnetite, black in color, was isolated by magnetic decantation, washed with distilled water and ethanol several times, and then, modified with aminosilane (APTS) [31–34]. For this aim, 25 ml of MNP-ethanol solution (5 g L^{-1}) was mixed with 125 ml ethanol and sonicated with ultrasonicator for 30 min. At the 20th min of sonication, 10 ml was added. After stirring mechanically for 15 h, the product was washed with methanol several times. APTS-coated MNPs, terminated with amine groups, were called “Generation 0” (G_0) [22,35]. In order to obtain first generation dendrimer, 200 ml of 20% (v/v) methylacrylate methanol solution was added to the APTS-modified MNPs. The suspension was immersed in an ultrasonication water bath at room temperature for 7 h. The particles were then rinsed with methanol five times by magnetic decantation. After rinsing, 40 ml of 50% (v/v) ethylenediamine methanol solution was added and the suspension was immersed in an ultrasonication water bath at room temperature for 3 h. The particles were rinsed with methanol five times by magnetic separation. For synthesizing the desired number of generations of dendrimers, stepwise growth using methylacrylate and ethylenediamine was repeated. The synthesized dendrimer-coated MNPs (DcMNPs) were dried, weighed and dispersed in PBS (5 g L^{-1}) [22,36,35].

2.3. Preparation and characterization of CpG-ODN/DcMNPs complexes

The study was carried out with the commercially available unmethylated ssDNA, CpG-ODN 2006 (K-ODN; also known as CpG-B). The CpG-ODN was dissolved in sterile endotoxin-free water to obtain a 50 μM stock solution, which was a clear and colorless solution after vortexing for complete solubilization. The stock solution was aliquoted and stored at −20 °C.

CpG-ODN/DcMNPs complexes were prepared by mixing equal amounts of the CpG-ODN solution and different amounts of