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In vitro and *in vivo* evaluation of anti-nucleolin-targeted magnetic PLGA nanoparticles loaded with doxorubicin as a theranostic agent for enhanced targeted cancer imaging and therapy

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ABSTRACT

A superparamagnetic iron oxide nanoparticles (SPIONs)/doxorubicin (Dox) co-loaded poly(lactic-*co*-glycolic acid) (PLGA)-based nanoparticles targeted with AS1411 aptamer (Apt) against murine C26 colon carcinoma cells is successfully developed via a modified multiple emulsion solvent evaporation method for theranostic purposes. The mean size of SPIO/Dox-NPs (NPs) was 130 nm with a narrow particle size distribution and Dox loading of 3.0%. The SPIO loading of 16.0% and acceptable magnetic properties are obtained and analyzed using thermogravimetric and vibration simple magnetometer analysis, respectively. The best release profile from NPs was observed in PBS at pH 7.4, in which very low burst release was observed. Nucleolin is a targeting ligand to facilitate anti-tumor delivery of AS1411-targeted NPs. The Apt conjugation to NPs (Apt-NPs) enhanced cellular uptake of Dox in C26 cancer cells. Apt-NPs enhance the cytotoxicity effect of Dox followed by a significantly higher tumor inhibition and prolonged animal survival in mice bearing C26 colon carcinoma xenografts. Furthermore, Apt-NPs enhance the contrast of magnetic resonance images in tumor site. Altogether, these Apt-NPs could be considered as a powerful tumor-targeted delivery system for their potential as dual therapeutic and diagnostic applications in cancers.

1. Introduction

Although the results of new strategies for cancer therapy provide satisfactory benefits for patients, the most of currently available cancer treatments are not curative mainly because of not being specifically delivered to the tumor site [1]. Most of the currently available drugs are not targeted which cause serious side effects necessitating the development of vehicles for targeted drug delivery to the malignant cells.

Dox-HCl ($C_{27}H_{30}ClNO_{11}$, MW: 579.99 g/mole), which is a weak amphipathic base is a highly hydrophilic drug (approximately 10 mg per 1 mL of water) that is derived by chemical semisynthesis from a bacterial species (wild type strains of *Streptomyces*) and wieldy used as chemotherapeutic agent [2,3]. As a DNA intercalating agent, Dox could effectively treat various types of cancers including hematological malignancies (like leukemia, blood cancers, and lymphoma), and several types of soft tissue sarcomas and carcinoma (solid tumors) [4–7]. The clinical application of Dox-HCl can be restricted due to its side effects, namely cardiotoxicity, myelotoxicity, nephrotoxicity and massive reactive oxygen species (ROS) generation [8,9]. Encapsulation of Dox into the hydrophobic polymers such as PLGA improves the chemotherapy outcome by enabling the safe dosing of Dox to overcome issues like rapid systemic clearance, cytotoxic effects or poor local distribution [10,11]. However, Dox is a highly hydrophilic drug, hence its

Abbreviations: SPIONs, superparamagnetic iron oxide nanoparticles; Dox, doxorubicin; PLGA, poly(lactic-*co*-glycolic acid); Apt, aptamer; NPs, nanoparticles; Dox-HCl, doxorubicin hydrochloride; MRI, magnetic resonance imaging; PBS, phosphate buffered saline; DLS, dynamic light scattering; AFM, atomic force microscopy; TEM, transmission electron microscope; FTIR, Fourier transformed infrared analysis; XRD, X-ray diffraction analysis; VSM, vibration sample magnetomer; TGA, thermogravimetric analysis; T1, relaxation time; emu/g, electromagnetic unit/gram.

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encapsulation into the PLGA is a challenging task. Nanobiotechnology is a new technology to overcome these drawbacks. Nanoparticle-based delivery systems are playing a crucial role to the treatment of cancers via a synchronous delivery of chemotherapeutic and diagnostic agents to the malignant cells [12,13]. The challenge lies in synthesis of new biocompatible and biodegradable nanocarriers which are capable of co-encapsulating nanoparticle-based contrast agents and chemotherapeutic drugs targeted with specific ligands in order to improve efficacy and tumor targeting properties [12,14,15]. There are many reports regarding the synthesis of nanocarriers such as PLGA, polylactic acid (PLA), polyethylenimine (PEI), polycaprolactone (PCL) and chitosan based on the combination of diagnostic agents and chemotherapeutic drugs for tumor targeted delivery [16–18]. Particle size is one of the most important parameters that determines both *in vitro* and *in vivo* fate of nanoparticle-based drug delivery systems [19,20]. It has been shown that nanocarriers with size ranging from 100 to 200 nm have favorable enhanced permeability and retention (EPR) within tumor vasculature [21,22]. Additionally, these nanoparticles should meet eligibility requirements of an excellent nanoparticle in terms of adequate systemic clearance, high capacity of drug loading and controlled drug release [6,23–25]. Clinical application of poly(lactide-*co*-glycolide)-b-poly (ethylene glycol)-carboxylic acid (PLGA-PEG-COOH), as a safe and biodegradable controlled release polymer system, has been well demonstrated [26,27]. It has significantly reduced systemic clearance which is attributed to poly(ethylene glycol) (PEG) compared with polymers without PEG [28,29].

AS1411 aptamer is a quadruplex-forming DNA strand that binds to nucleolin [30,31]. Nucleolin proteins are overexpressed in the cytoplasmic membrane of malignant and endothelial cells [32,33] and play an important role as specific proteins for binding to variety of ligands that are responsible for the cell proliferation and adhesion [34,35]. It has been also showed that nucleolin has an important role in angiogenesis, thus the application of AS1411-mediated drug delivery could meet the requirements for promising dual targeting strategy in terms of depriving tumor cells from nutrient supply [34]. Gao et al. demonstrated that AS1411-targeted nanoparticles enhanced the penetration through monolayers cancer cells, which was valuable for reaching to the part of solid tumors that lacked microvessels, performing a more specific and effective anti-tumor effects [36]. In this contribution, SPIO/Dox co-encapsulated nanoparticles were synthesized and then conjugated to AS1411 aptamer as a dual therapeutic and diagnostic strategy for targeted anti-tumor drug delivery. Endorem® and Resovist® were among several SPIONs products that were used as contrast agents in clinical diagnosis. They were not coated with either suitable materials or tumor-specific moieties causing their rapid uptake by RES [37,38]. Therefore, they were withdrawn from the market years ago due to the lack of impact.

In this study, a co-precipitation method was used to synthesize the SPIONs as diagnostic agents [39,40]. Then, SPIONs and Dox were entrapped in the PLGA-based nanoparticles via a modified multiple emulsion solvent evaporation method [41] and AS1411 aptamer was conjugated to the surface of NPs through an EDC/NHS technique [22]. Different physico-chemical properties of the prepared formulations, including particle size distribution, drug loading capacity and saturation magnetization were characterized [42,43]. In this study, we also investigated the presence of nucleolin on the surface of murine C26 cancer cells by an immunocytochemistry test. Cellular uptake and cytotoxicity effects of nanoparticles were determined and were confirmed by flow cytomerty analysis, fluorescence microscopy, Prussian blue staining and cytotoxicity test. *In vivo* biodistribution and anti-tumor efficacy were carried out on mice bearing C26 colon carcinoma to evaluate the tumor targeting efficacy of nanoparticles. Many researchers have synthesized SPIONs by different methods followed by coating them with suitable materials and tumor-specific ligands to evaluate the contrast enhancement efficacy of SPIONs *in vitro* and *in vivo* by MRI technique [44–46]. In this contribution, MRI was used to evaluate the potential of synthesized nanoparticles as MRI contrast agents for diagnostic applications, as well.

2. Materials and methods

2.1. Materials

Dox-HCl, Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$, 99%), Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$, 99%), ammonium hydroxide (5 M) and Prussian blue kit was purchased from Sigma Aldrich (USA). Poly(lactide-*co*-glycolide) with terminal carboxylate groups (PLGA-COOH, Resomer RG503H, lactic/glycolic acid ratio (LG) 50:50, MW: 35 kDa) and poly(lactide-*co*-glycolide)-b-poly (ethylene glycol)-carboxylic acid endcap (PLGA-PEG-COOH, LG 50:50, M_w: 40:5 KDa) were obtained from Boehringer Ingelheim (Germany) and Akina, Inc (USA), respectively. Rabbit polyclonal primary antibody to nucleolin, Goat anti-rabbit IgG H&L (conjugated to horseradish peroxidase or HRP) secondary antibody and 3,3′-diaminobenzidine (DAB) chromogen kit was obtained from Abcam Biotechnology, Inc (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (USA). AS1411 DNA aptamer (Apt, sequence: 50-TTGGTGGTGGTG GTTGTGGTGGTGGTGG-30, 28 bp) was synthesized by a DNA synthesizer apparatus (Polygene, Germany). Roswell Park Memorial Institute 1640 (RPMI1640) culture medium, fetal bovine sera (FBS), trypsin and penicillin–streptomycin solution were obtained from Gibco (Germany). All chemicals and solvents were of analytical grade.

BALB/c mice and murine C26 colon carcinoma cell line were obtained from the National Cell Bank and Laboratory Animal Resources Center at the Pasteur Institute of Iran and treated according to the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences guidelines.

2.2. Synthesis of SPIONs

The synthesis of SPIONs has been described by Jain and coworkers using a co-precipitation method [39]. Briefly, Fe (III) chloride (0.1 M, 30 mL) and Fe (II) chloride (0.1 M, 15 mL) were mixed in deoxygenated distilled water with nitrogen (N_2) for 15 min. NH_4-OH solution (5 M, 3 mL) was added dropwise and stirred for 15 min under nitrogen atmosphere at 25 °C. When the color of solution turned to deep black, 250 mg oleic acid was added dropwise into the suspension and heated up to 85 °C for 30 min with continuous stirring to evaporate the ammonia. Finally, the resulting black precipitate was separated by applying an external magnetic field, washed two times with 15 mL of distilled water and freeze dried for 2 days at −60 °C under 7 μm Hg vacuum. A chloroform solution of lyophilized SPIONs (30 mg/mL) was sonicated in an ultrasonic bath for 5 min at 200 W (W) and was centrifuged (14000*g*, 10 min) to further remove very small aggregates and undispersed residues. The resulting SPIONs were stored at 4 °C until use.

2.3. Preparation of NPs

NPs were prepared by using a modified multiple emulsion solvent evaporation method [41]. Firstly, 1 mL chloroform solution of SPIONs (5 mg/mL) was prepared as a primary organic phase (O_1)