Journal of Pharmaceutical Sciences xxx (2017) 1-8

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Nanotechnology

Environmentally Responsive Dual-Targeting Nanoparticles: Improving Drug Accumulation in Cancer Cells as a Way of Preventing Anticancer Drug Efflux

Cenk Daglioglu*

Department of Molecular Biology and Genetics, Faculty of Science, Izmir Institute of Technology, Urla, Izmir 35430, Turkey

ARTICLE INFO

Article history:
Received 5 June 2017
Revised 15 October 2017
Accepted 17 October 2017

Keywords:
nanoparticles
conjugation
drug resistance
targeted drug delivery
responsive delivery systems

ABSTRACT

Drug targeting and stimuli-responsive drug release are 2 active areas of cancer research and hold tremendous potential in the management of cancer drug resistance. In this study, I addressed this issue and focused on the synthesis and characterization of pH-responsive Fe_3O_4@SiO_2(FITC)-BTN/folic acid/DOX multifunctional nanoparticles aiming to increase drug accumulation in malignancies with both dual active targeting and endosomal drug release properties. Dye-doped silica magnetic-fluorescent composite was constructed by a simple coprecipitation of Fe^{+2}/Fe^{+3} salts followed by sol-gel formation and dual-targeting function was obtained by conjugating folate and biotin moieties on the silica surface of nanoparticles via an esterification reaction. Doxorubicin was then successfully attached on the amine-functionalized nanoparticles using a pH-sensitive Schiff-base formation. The physicochemical characterization of the structure was performed by dynamic light scattering, zeta potential measurement, X-ray diffraction, Fourier transform infrared spectroscopy, electron microscopy techniques, and an *in vitro* pH-dependent release study. Cellular uptake and cytotoxicity experiments demonstrated an enhanced intracellular delivery and reduction of cancer cell viability in the cervical carcinoma HeLa cell line. Furthermore, proapoptotic studies showed that the nanoparticles increased the apoptotic rates within the same cancer cells. The preliminary cell tests confirm the potential of these multifunctional nanoparticles against the development of drug resistance in cancer cells.

Introduction

Drug resistance is one of the main problems in clinical cancer treatment. The overexpression of drug efflux transporters, such as P-glycoprotein (P-gp), plays a significant role in the progression of resistance.[1] To achieve targeted therapeutic concentration, higher doses or frequency of dosing are required but thus resulting in greater toxicity.[2] In order to address this problem, considerable attempts have been made to exploit the drug delivery systems (DDSs) for overcoming drug resistance in cancer therapy.[3] Although most DDSs conferred improved pharmacokinetics and biodistribution profiles, the poor cellular uptake and insufficient drug release inside cells remain rate-limiting steps, which lead to low intracellular drug concentration below the therapeutic window.[4] Therefore, both active targeting capacity and environmentally responsive drug release character are essential features for an ideal DDS to evade drug pump efflux activity as a way of preventing drug resistance.

Among the numerous DDSs, magnetic (Fe_3O_4) nanoparticles are promising as drug delivery vehicles for both diagnostic and therapeutic applications.[5] The ease of the synthesis of magnetic nanoparticles and subsequent incorporation of various cell-specific targeting, imaging, and therapeutic functions have enabled these systems to be employed as smart nanomedicines.[6] In this regard, DDSs endowed with the abilities to deal with efficient accumulation and drug release in cancer cells could lead to easier reaching of anticancer drug to the effective therapeutic concentration before the development of drug resistance.

Vitamin receptors are upregulated on a variety of human cancers due to their enhanced mitosis rates. Therefore, the overexpression of these receptors can be exploited to target vitamin-linked DDSs specifically to tumor cells.[7] Considering the results reported in our previous works, the nanoparticles vectorized with biotin (vitamin B7)[8] or folate (FA) (vitamin B9)[9,10] were successfully penetrated into the cancer cells via receptor-mediated endocytosis. In this study, to further enhance the accumulation level of nanoparticles and to promote fast intracellular drug release

Conflicts of interest: The author reports no declarations of interest.

* *Correspondence to:* Cenk Daglioglu (Telephone: +90 232 750 7319; Fax: +90 232 750 7303).
E-mail address: cenkdaglioglu@iyte.edu.tr (C. Daglioglu).

https://doi.org/10.1016/j.xphs.2017.10.029
0022-3549/

in cancer cells in order to prevent cellular efflux, the effect of the coconjugation of 2 active targeting ligands on the surface of the nanoparticles in combination with pH-dependent drug release property was investigated. For this reason, pH-responsive 3 successive nanoparticles: (1) Fe_3O_4@SiO_2(FITC)-BTN/DOX nanoparticles containing only biotin; (2) Fe_3O_4@SiO_2(FITC)-FA/DOX nanoparticles containing only folate; (3) Fe_3O_4@SiO_2(FITC)-BTN/FA/DOX nanoparticles containing both biotin and folate were fabricated to evaluate accumulation potential of the nanoparticles between single and dual targeting in inherently drug-resistant HeLa cells, which conditionally overexpress 21 of the 36 investigated transporters.[11]

Material and Methods

Materials

Iron (II) chloride tetrahydrate ($FeCl_2$ $4H_2O$) (99%), iron (III) chloride hexahydrate ($FeCl_3$ $6H_2O$) (98%), tetraethyl orthosilicate 99.9%, fluorescein isothiocyanate (FITC), biotin (BTN), FA, 3-aminopropyltriethoxysilane (APTES), N,N-dicyclohexyl-carbodiimide, N-hydroxysuccinimide, glutaraldehyde 25% aqueous solution, FT-IR grade potassium bromide ≥99% (KBr), dimethyl sulfoxide (DMSO), Triton X-100, 3-(4,5-dimethyl-2-thialzolyl)-2,5-diphenyltetrazolium bromide (MTT), and trypsin were purchased from Sigma-Aldrich Chemicals. Doxorubicin was obtained from SABA Pharma. Oleic acid (99%), ammonium hydroxide 25% aqueous solution, 1-hexanol (>98%), cyclohexane, and toluene were purchased from Fluka/Riedel-de Haën Chemicals. 7-aminoactinomycin (7-ADD) and PE-annexin-V were purchased from BD Biosciences. Dulbecco's Modified Eagle Medium growth medium, 10% fetal bovine serum, streptomycin, penicillin, and L-glutamic acid were purchased from Gibco Life Technologies. All other chemicals and reagents were of the highest purity. All the experiments were performed in deionized Milli-Q water.

Cell Cultures

HeLa (human epithelial cervical carcinoma) (93021013) cell line was kindly provided by the Biotechnology and Bioengineering Research and Application Centre, Izmir Institute of Technology, Turkey. The cancer cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamic acid. The cell line was incubated in 5% CO_2 and 90%-100% relative humidity at 37°C. Medium renewal was carried out 2 to 3 times per week, and cells were subcultured when they achieved 80%-90% confluence. The cell line was discarded after 20 generations, and new line was obtained from frozen stocks.

Synthesis of Multifunctional Nanoparticles

The parental Fe_3O_4@SiO_2(FITC) nanoparticles were synthesized as described in our previous work.[9] They consist of superparamagnetic iron oxide nanoparticles (as magnetic contrast agent), coated with layers of silica shells, encasing FITC within (as optical contrast agent), for imaging, biocompatibility, and molecular functionalization. To ensure preferential tumor cell uptake of the nanoparticles, the outermost layer of Fe_3O_4@SiO_2(FITC) was then functionalized with BTN and/or FA molecules by silanization with BTN-APTES and/or FA-APTES conjugate. In brief, an APTES ester of BTN and/or FA (BTN-APTES/FA-APTES) was prepared by mixing biotin (8.0 mg) and/or folate (4.0 mg) with APTES (2.0 μL) in 40 mL dry DMSO in the presence of N-hydroxysuccinimide (1.1 mg) and N,N-dicyclohexyl-carbodiimide (4.7 mg) as the catalyst at room temperature for 2 h. After this, a mixture of Fe_3O_4@SiO_2(FITC) nanoparticles (100 mg), BTN-APTES and FA-APTES conjugate, and free APTES (17 μL) in toluene (160 mL) was stirred at room temperature for 24 h to introduce BTN-APTES and FA-APTES conjugate and free APTES on the surface of silica-coated nanoparticles by hydrolysis and condensation of APTES through silanization. Final products were collected by a magnet, washed with toluene and ethanol several times to remove any unreacted reactants, and dried in vacuum oven at room temperature, overnight. In this step, besides vectorization of nanoparticles, simultaneously the surfaces were modified with free APTES to form an amine-terminated overlayer for further functionalization.

Fe_3O_4@SiO_2(FITC)-BTN/DOX, Fe_3O_4@SiO_2(FITC)-FA/DOX, and Fe_3O_4@SiO_2(FITC)-BTN/FA/DOX nanoparticles were prepared by conjugating DOX on the surface of Fe_3O_4@SiO_2(FITC)-BTN/NH_2, Fe_3O_4@SiO_2(FITC)-FA/NH_2, and Fe_3O_4@SiO_2(FITC)-BTN/FA/NH_2 nanoparticles via glutaraldehyde activation, respectively. For pH-responsive drug release, DOX complex was covalently linked to amine-functionalized silica surface of nanoparticles via pH-labile Schiff-base formation from the amino sugar moiety of DOX.[12] This acid-sensitive linkage is stable at natural pH (~7.4), but broken at mildly acidic pH (~5.0), which allows for the release of DOX in the more acidic endosome environment (pH 5.0) versus systemic circulation pH (7.4). Briefly, the surface of Fe_3O_4@SiO_2(FITC)-BTN/NH_2, Fe_3O_4@SiO_2(FITC)-FA/NH_2, and Fe_3O_4@SiO_2(FITC)-BTN/FA/NH_2 nanoparticles (10 mg) was activated in 20 mL 1.0% glutaraldehyde solution under vigorous mechanical stirring at room temperature for 1 h. Then, nanoparticles were collected via centrifugation, and the unreacted glutaraldehyde was removed by extensive washing with ultrapure water. Glutaraldehyde-activated nanoparticles were subsequently incubated with DOX complex (10 μM) in 20 mL phosphate-buffered saline (PBS) solution (pH 7.4) under vigorous mechanical stirring at room temperature for 6 h. The amount of bound DOX was calculated from the difference between the amount of DOX introduced into the coupling reaction mixture and the amount of DOX present in the washing water after immobilization by measuring DOX absorbance at 480 nm. The resulting nanoparticles: (1) Fe_3O_4@SiO_2(FITC)-BTN/DOX; (2) Fe_3O_4@SiO_2(FITC)-FA/DOX; and (3) Fe_3O_4@SiO_2(FITC)-BTN/FA/DOX were magnetically separated and washed with 1% DMSO in PBS several times to remove any unreacted reactants and dried under vacuum at room temperature, overnight.

Structural and Physicochemical Characterization

Dynamic light scattering (DLS) measurements were performed at 25°C, using a Malvern Zetasizer Nano ZS compact scattering spectrometer. Average hydrodynamic diameters, size distributions, and surface charge analysis of the samples were determined using Malvern Dispersion Technology Software 7.11. Nanoparticles were suspended in ultrapure water to give optimum signal intensity. All measurements were repeated 5 times to verify the reproducibility of the results.

Powder X-ray diffraction (XRD) measurements were performed with "Philips X'Pert Pro," at room temperature by using CuKα radiation (λ = 1.5405 Å) and Bragg–Brentano θ/2θ configuration. The measurements were performed over the 2θ range of 20-70°.

The FTIR spectroscopy spectra of the nanoparticles were collected with a "PerkinElmer Spectrum-100" spectrophotometer in the range 450-4000 cm^{-1}. The spectra of the dried samples were obtained by employing a KBr pellet.