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Formulation and evaluation of targeted nanoparticles for breast cancer theranostic system

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ABSTRACT

Theranostic polymeric NPs developed for both cancer diagnosis and cancer therapy. This multifunctional polymeric vehicle was prepared by a single emulsion evaporation method, using carboxyl-terminated PLGA. LHRH as a targeting moiety, was conjugated to the surface of polymeric carrier by applying polyethylene glycol. The results indicated that the diameter of NPs was ~185.4 ± 4.6 nm as defined by DLS. The entrapment efficacy of docetaxel, silibinin, and SPIONs was 84.6 ± 4.1%, 80.6 ± 2.7%, and 77.9 ± 4.3%, respectively. The NPs showed a triphasic in-vitro drug release pattern. MTT assay was done on two cell lines, MCF-7 and SKOV-3. Enhanced cellular uptake ability of the targeted NPs to MCF-7 was evaluated in-vitro by confocal laser scanning microscopy. The results indicated that compared to non-targeted NPs, the LHRH targeted NPs had significant efficacy at IC50 concentration. The effect of the NPs on VEGF expression in MCF-7 and SKOV-3 cells was investigated by Real-Time PCR method. VEGF mRNA level expression in MCF-7 cell line reduced by 83% in comparison to control cell line. The designed NPs can be used as promising multifunctional platform for detection and targeted drug delivery in breast cancer.

1. Introduction

Breast cancer is the main cause of death among women. In 2015, of about 231,840 cases of invasive breast cancer, and 40,000 deaths cases have been reported. In addition to invasive breast cancer in 2015, approximately 60,000 newly diagnosed cancer cases were pertaining to women (DeSantis et al., 2016). Among different methods of breast cancer treatment chemotherapy is most effective one (Lyman, 2015). Anthracycline-based medicines and taxanes (paclitaxel and docetaxel) are now used as first-line chemotherapy treatment for metastatic breast cancer (MBC). The targeted delivery of the chemotherapeutic drugs can significantly decrease their toxic side effects on normal cells (Jabarian et al., 2013; Taheri et al., 2012b).

Multifunctional nano-carriers accompanying with contrast agents (e.g., magnetic nanoparticles), are proposed as promising platforms for targeting drug delivery and monitoring the different cancerous tumor (Azhdarzadeh et al., 2016; Mahmoudi et al., 2011). Recent developments include the design of new nanoparticle-based MRI contrast agents. So that the magnetic NPs are coated with a suitable material and conjugated to specific ligands for improved performance and functionality of drug delivery system (Khafaji et al., 2016; Sharifi et al., 2015). Different polymers have been used such as SPIONs loaded chitosan for magnetic targeting drug delivery (Mahmoudi et al., 2011; Rose et al., 2016). But these systems have some general objections that is low capacity of drug loading and immediately are cleared after intravenous administration (Bakhtiary et al., 2016; Laurent et al., 2014).

Peptide receptors are widely expressed in tumor cells, such as, they enhance targeting drug delivery (Talaei et al., 2013). LHRH is a decapeptide hormone secreted by the hypothalamus gland and regulates reproduction. LHRH receptors are widely expressed on the surface of tumor cells, including cancers of the ovary, breast, prostate, and endometrial. Vice versa, the LHRH receptors, are less expressed on the surface of normal cells (Abashzadeh et al., 2011). So LHRH can be used as targeting moiety in LHRH-receptor-positive cancer cells including breast cancer for improving treatment efficiency (Li et al., 2015;

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Nukolova et al., 2013). This can improve the cytotoxicity of NPs on LHRH-receptor-positive tumor cells, MCF-7 cells (Taheri et al., 2012a) and LHRH-receptor-negative cells, SKOV-3 cells (Taratula et al., 2016) in-vitro.

Polylactide-*co*-glycolide (PLGA) is biodegradable polyester that has been recommended for human consumption (Dadras et al., 2011; Varshochian et al., 2013). It can be entrapted the different drug molecules or bounded to them with covalent attachment (Noori Koopaei et al., 2014). Docetaxel (DTX) is an effective toxoid for microtubule depolymerization that has been widely used against breast, gastric, pancreatic, and urothelial carcinomas (Nateghian et al., 2016). Silibinin (SIL), a main constituent (flavonolignan) of the fruits of *Silybum marianum*, (milk thistle plant), is used as herbal medicinal. It has several properties, including inhibition of stem cell proliferation, tumor cell cycle disorders, induction of apoptosis in cancer cells such as breast cancer and breast cancer stem cells (Mahmoodi et al., 2014).

Vascular endothelial growth factor (VEGF) is a type of growth factor that functions as a signaling protein. It is very effective in detecting angiogenesis and metastasis. VEGF receptors in response to extracellular stimuli such as inflammation, hypoxia, cytokines, etc., are widely expressed on the surface of tumor cells (Amara et al., 2016).

Our goal in this research was to assess how the targeted ligand (LHRH) and diagnostic agent (SPIONs) perform in the treatment and diagnosis of breast cancer. Our experiments have shown the increase in many in-vitro characteristics due to use of LHRH.

2. Materials and methods

2.1. Cell lines, culture conditions, reagents, and instruments

This experiments required following materials from re-known centers: Poly (ethylene glycol) (PEG, Mw: 3400&2000), Carboxyl-terminated poly (D, L-lactide-*co*-glycolide) (PLGA-COOH, L: G molar ratio: 50:50, Mw: 40,000–75,000), and docetaxel (DTX) were purchased from Sigma Co. (USA). Maleimide poly (ethylene glycol) – NHS ester (mal-PEG-NHS ester, Mw: 3400) was obtained from Jenkem Co. (USA). TPGS (Vitamin E D-α-tocopherol polyethylene glycol 1000 succinate), iron (III) chloride ($FeCl_3 \cdot 6H_2O$), iron (II) chloride ($FeCl_2 \cdot 4H_2O$), CTAB (cethyl trimethyl ammonium bromide) and 1-butanol were obtained from Sigma Chemical Co (USA). Luteinizing hormone-releasing hormone human (LHRH) was obtained from Cinagen Co. (Iran). Iran Pasteur Institute provided us cell culture media and fetal bovine serum (FBS). Breast cancer cell line, MCF-7 and Ovary cancer cell line, SKOV-3 was purchased from Pasteur Institute (Iran). HPLC grade acetonitrile, methanol, DMSO, HCl, NH_4OH, toluene, acetone, and other analytical grade chemicals were purchased from Merck (Germany).

By using DLS (Nano/Zeta sizer-ZS 90, Malvern Instrument, United Kingdom), the zeta potential and size of the NPs were measured. The NPs morphology was evaluated by Nova Nano SEM 430 (Netherlands) and by Hitachi H-7650 TEM (Japan). The nanoparticles' infra-red spectra were studied by using MAGNA 760 FT-IR, Nicolet Instrument, USA, with spectroscopic grade KBr (Supplementary materials). To understand the nature of the NPs crystallinity X-ray diffraction device was used (XRD, X'pert 1710, Philips, Germany) at room temperature (RT) using CuKα ($\alpha = 1.54056$ Å) in range of 2θ. A Vibrating Sample Magnetometer (VSM-BS2-11 Tesla, Kashan University Laboratory) recorded the magnetic properties of the NPs, at 10 K and 300 K. Protein corona effects was carried out by gel electrophoresis using SDS-page method (Behzadi et al., 2014; Mao et al., 2013). The DSC spectra of the NPs were taken using DSC, Mettler-Toledo 822e Switzerland. The medicines (DTX and SIL) and SPIONs loading contents of the NPs were determined in triplicate by HPLC (Prominence LC-20A, C18 column, Shimadzu, Japan) and AAS (Z-2000, Hitachi, Japan) respectively.

2.2. Synthesis of DTX/SIL/SPION@LHRH functionalized PLGA NPs

2.2.1. Synthesis of SPIONs

The synthesis of SPIONs was similar to previous research (Panahifar et al., 2013).

2.2.2. Synthesis of NH_2-PEG-SH

NH_2-PEG-SH was synthesized from poly ethylene glycol (PEG) by applying the synthetic method used by Manjili et al. (2016). Full process has taken up in Supplementary materials section.

2.2.3. Synthesis of DTX/SIL/SPION@LHRH functionalized PLGA NPs

The synthesis of DTX/SIL/SPION@LHRH functionalized PLGA NPs was carried out in three stages. The first step was the preparation of polymeric NPs containing three components of DTX/SIL/SPIONs by the single emulsion/solvent evaporation method (Ebrahimnezhad et al., 2013). First, a solution was made by dissolving 10 mg DTX in 2 mL dichloromethane (DCM). Then 25 mg SPIONs and a polymeric solution (100 mg PLGA in 3 mL DCM) were added to the mixture separately. Finally for finding the optimum amount of SIL, three different amounts 20, 40, and 60 mg SIL were dissolved into acetone, and by adding each solution to the above mentioned mixture, three different organic phase were formed (Ebrahimnezhad et al., 2013; Sadiq and Rassol, 2014). The organic phase was added slowly to the aqueous phase containing 0.5% (w/v) PVA and 0.1% (w/v) TPGS and emulsified by using probe sonicates (130 W/5 min). The oil-in-water emulsion was stirred overnight (600 rpm/RT) for solvent evaporation. Finally triple DTX/SIL/SPIONs-loaded PLGA NPs were formed. The nanoparticles were centrifuged and washed three times for purification (1200 rpm) and then it was freeze dried.

The second step was modification of NPs with synthesized NH_2-PEG-SH. 5 mmol of triple DTX/SIL/SPIONs-loaded PLGA NPs was incubated with EDC/NHS (100 μL 50 mg/mL, 20 min/RT). NHS-activated NPs through covalent bond were attached to 5 mmol NH_2-PEG-SH (24 h/300 rpm/RT) to form PEG modified NPs (DTX/SIL/SPIONs@PLGA-NH-PEG-SH).

In the final step, LHRH (5 mmol in 1 mL D/W) bearing free amine groups was conjugated to mal-PEG-NHS (5 mmol) and formed mal-PEG-LHRH. Then DTX/SIL/SPIONs@PLGA-PEG-SH NPs was conjugated with mal-PEG-LHRH (EDC/NHS 50 mg/mL), stirred overnight (300 rpm/4 °C). The DTX/SIL/SPION@LHRH functionalized PLGA NPs were purified using dialysis bag (Mw cutoff 12KD).

2.3. Identification and determination of LHRH on the surface of NPs

The DSC instrument was calibrated with indium and zinc. 10 mg of sample was placed in an aluminum enclosure, sealed and under nitrogen flow was heated at 10 °C/min from 20 to 300 °C. The samples consist of LHRH, DTX/SIL/SPIONs@PLGA NPs; DTX/SIL/SPION@LHRH functionalized PLGA NPs and physical mixture of LHRH and DTX/SIL/SPIONs@PLGA NPs.

The Bradford reaction was used to measure protein concentration (Zhao et al., 2014). Absorption of samples was read at 595 nm. The amount of LHRH bonded to polymeric NPs was obtained by subtracting the total LHRH used in the synthesis from not bonded LHRH.

2.4. DTX, SIL and SPIONs loading contents and in-vitro drug release

The drugs and SPIONs content was calculated by the following formula:

SPIONs content: weights of the iron oxide in NPS/weight of NPs

$$\text{Drug loading } (\%) = \frac{\text{weight of drug in particles}}{\text{weight of particles}} \times 100$$