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Epithelial cell adhesion molecule aptamer conjugated PEG–PLGA nanopolymersomes for targeted delivery of doxorubicin to human breast adenocarcinoma cell line *in vitro*

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ABSTRACT

Targeted delivery of anti-cancer agents exclusively to tumor cells introduces an attractive strategy because it increases the therapeutic index compared with untargeted drugs.

Aptamer conjugated nanoparticles that can specifically bind to the proteins on a tumor cell surface are capable nanoscale delivery systems for enhancing cellular uptake of chemotherapeutic agents. The epithelial cell adhesion molecule (EpCAM) as a cancer stem cell marker emerges as a versatile target for aptamer-based cancer therapy due to its high expression level in various adenocarcinoma cell lines and its very low expression level in normal cells.

We developed EpCAM-targeted PEG–PLGA nanopolymersomes by covalently coupling the EpCAM aptamer to the surface of nanopolymersomes loaded with the anticancer agent doxorubicin via pH gradient method.

The results indicated that doxorubicin was entrapped in PEG–PLGA nanopolymersomes with encapsulation efficiency and loading content of $91.25 \pm 4.27\%$ and $7.3 \pm 0.34\%$, respectively. Over a period of 5 days, up to 8% of the DOX was released through this system.

The doxorubicin-loaded aptamer conjugated nanopolymersomes exhibited efficient cell uptake and internalization, and were significantly more cytotoxic ($P < 0.01$) toward EpCAM-positive tumor cells (MCF-7) than non-targeted nanopolymersomes. Our data suggest that EpCAM-targeted nanopolymersomes will lead to an improved therapeutic index of doxorubicin to EpCAM positive cancer cells.

1. Introduction

As a major treatment of cancer, chemotherapy is often far from perfect, with undesirable associated side effects such as systematic toxicity. The reduced systematic toxicity of targeted anti-cancer drug delivery systems can be due to enhanced delivery of chemotherapeutics to the tumor tissue. Nanocarriers can significantly intensify the anti-tumor efficiency of various chemotherapeutic agents according to passive tumor-targeting effects of enhanced permeability and retention (EPR) (Yan et al., 2014; Song et al., 2014; Levacheva et al., 2014). Furthermore, through conjugation of a targeting agent to a nanoparticle surface, an active targeting effect could be established.

Previously, monoclonal antibodies were implemented as targeting ligands (Amin et al., 2013). In the past decade, peptides (Oh et al., 2014a), aptamers (Lu et al., 2014), and chemical molecules (Cho, 2015) have been introduced as the new generation of targeting agents. Aptamers are small strands of DNA or RNA, which have a unique folding, which specifically bind to the target molecule with high affinity.

The SELEX, a repetitive *in vitro* process of sequential selection and amplification steps, produces specific aptamers with a high affinity for a desired target (Chai et al., 2011; Szeitner et al., 2014).

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In addition to elegant structural and molecular characteristics, aptamers are easily modified by chemical reactions, and compared to antibodies, induce minimal immune responses *in vivo* (Jayasena, 1999).

Previously, various aptamer-conjugated carriers have been investigated as targeted drug delivery for cancer therapy (McConnell et al., 2014; Zhu et al., 2014). Promising results were achieved with aptamer conjugated nanoparticles (NPs) targeting mucin-1 (MUC-1), HER2/neu, epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule (EpCAM), nucleoin, and prostate-specific membrane antigen (PSMA) over-expressed on the surface of different tumor cell lines (Chen et al., 2014; Liu et al., 2012; Taghdisi et al., 2013; Tan et al., 2013; Das et al., 2014; Li et al., 2014a).

It was proved that aptamer-targeting drug delivery successfully enhances the therapeutic effect of anti-cancer agents on prostate cancer, breast cancer, colorectal cancer, pancreatic cancer, hepatic cancer, and lymphoblastic leukemia (Li et al., 2014b; Zhou et al., 2014; Ray et al., 2012; Shahidi-Hamedani et al., 2013).

The aforementioned studies have paved the way to develop aptamer-conjugated nanoparticles as the targeted drug delivery systems. With the new strategies for coupling of specific ligands to the surface of NPs (Li et al., 2014d; Oh et al., 2014b), and owing to successful experiments in aptamer targeting, there is a demand in the development of aptamer-guided NPs for targeted delivery of anticancer agents to cancerous cells *in vitro* and *in vivo* (Dassie et al., 2014).

The epithelial cell adhesion molecule (EpCAM) is a glycosylated transmembrane protein, which induces cell growth by the persistent expression of the activated c-myc oncogene (Litvinov et al., 1997). Moreover, EpCAM is involved in Ca^{2+}-independent cell adhesion, is highly expressed in many solid tumors, and exhibits a low level of expression in normal healthy tissues (Winter et al., 2007; Gires and Stoecklein, 2014). In breast cancer, EpCAM overexpression is associated with metastatic progression, tumor relapses, and, consequently, poor overall survival (Tas et al., 2014; Massoner et al., 2014). Accumulating evidence suggests EpCAM positive tumor cells are tumor initiating cells with stem or progenitor cell features in colorectal, breast, and pancreatic cancer (Schulze et al., 2013).

Until now, aptamer-guided nano-carriers for delivery of therapeutic quantities of doxorubicin to cancer stem cells have not been reported in the literature. The 19-mer EpCAM RNA aptamer was demonstrated to specifically bind to the extracellular domain of the epithelial cell adhesion molecule. Other investigators successfully used conjugation of this targeting ligand directly to doxorubicin in order to deliver doxorubicin to MCF-7 cells (Subramanian et al., 2012).

Here, we developed EpCAM-specific PEG–PLGA nanopolymersomes efficiently loaded with the doxorubicin and surface conjugated with the stable, high-affinity single-strand EpCAM RNA aptamer. This polymeric drug delivery system was characterized for cellular uptake, internalization, and cytotoxicity *in vitro*.

2. Materials and method

2.1. Materials

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) (Average M_w: 10,000 Da; lactic acid: glycolic acid = 75:25), *N*-hydroxysulfosuccinimide (NHS), 1-ethyl3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich (Schnelldor, Germany). The heterofunctional PEG polymer with a terminal amine and carboxylic acid functional groups (HCl·NH_2–PEG–COOH, M_w: 3500) was purchased from JenKem Technology, USA (Beijing, China). Doxorubicin hydrochloride (DOX) was purchased from Euroasia Co., Ltd. (Delhi, India). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were purchased from GIBCO (Darmstadt, Germany). Other solvent and chemical reagents were procured from Merck (Darmstadt, Germany) without further purification.

An anti-EpCAM RNA aptamer reported previously (Shigdar et al., 2011) was employed as the targeting ligand. The 19-mer EpCAM RNA aptamer (sequence: 5′-amino-C6 linker-–A(2′-F-C)G (2′-F-U)A(2′-F-U) (2′-F-C) (2′-F-C) (2′-F-C) (2′-F-U) (2′-F-U) (2′-F-U) (2′-F-U) (2′-F-C)G(2′-F-C) G(2′-F-U)-3′; where *F* = 2′-fluoro, molecular weight: 6345) with and without 3′-fluorescein modification was custom synthesized by MicroSynth (Balgach, Switzerland).

2.2. Cell lines

Human breast adenocarcinoma cell lines MCF-7 (C135) and MDA-MB231 (C578) were obtained from the National Cell Bank of Iran, Pasteur Institute of Iran. The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS and penicillin/streptomycin (100 U/mL, 100 units/mL) at 37 °C in a humidified atmosphere (95%) containing 5% CO_2.

2.3. Synthesis and characterization of PEG–PLGA copolymer

One gram of PLGA–COOH (10 kDa M_w, Sigma Aldrich) in 4 mL dichloromethane was gently stirred at room temperature in the presence of *N*-hydroxysuccinimide (1:8 PLGA:NHS molar ratio) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (1:8 PLGA: EDC molar ratio) to prepare PLGA–NHS. PLGA–NHS was precipitated with cold diethyl ether and washed three times with cold freezing solution containing 80% diethyl ether and 20% methanol to remove residual NHS and EDC.

After drying under a vacuum, PLGA–NHS was dissolved in chloroform (5 mL) followed by the addition of HCl·NH_2–PEG–COOH (1:1.2 PLGA:PEG molar ratio) and *N*,*N*-diisopropylethylamine (0.2 mmol). The co-polymer was precipitated with cold diethyl ether after 24 h and washed with methanol:diethyl ether solution (30%:70%). The final PLGA–PEG block co-polymer was freeze dried for 48 h and stored at −20 °C until use.

The ^{1}H NMR spectra of the PEG–PLGA copolymer in deuterated chloroform was recorded at room temperature using a Bruker Avance 400 MHz NMR spectrometer (Rheinstetten, Germany) to verify PEG conjugation to PLGA.

Molecular weights and polydispersity of PEG–PLGA copolymer was determined using the Agilent GPC-Addon system and RID-A refractive index signal detector recording at 212 nm coupled to the PLgel columns and operated at temperature 25 °C. The molecular weights were calibrated with polystyrene standards. Tetrahydrofuran was used as eluent (flow 1 mL/min), and the sample injection volume was 10 μL.

Thermal transitions of copolymers were determined using DSC (Mettler Toledo DSC 822, Switzerland). The sample size was approximately 2 mg, and the sample was subjected to thermal cycles from 0 to 90 °C at a rate of 10 °C/min.

2.4. Preparation DOX loaded nanopolymersomes

Doxorubicin loaded polymersomes were prepared using the pH gradient method (Bolotin et al., 1999). For this purpose, the thin dried polymer film of copolymer (20 mg) was hydrated using 2 mL aqueous ammonium sulfate 250 mM at 55 °C (above T_g of the copolymer) under 1250 rpm continuous stirring overnight. The polymersome dispersion was extruded 15 times at 55 ± 2 °C