Colloids and Surfaces B: Biointerfaces 148 (2016) 85–94

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Amine-functionalized poly(lactic-co-glycolic acid) nanoparticles for improved cellular uptake and tumor penetration

Song Yi Lee, Hyun-Jong Cho*

College of Pharmacy, Kangwon National University, Chuncheon, Gangwon 24341, Republic of Korea

ARTICLE INFO

Article history:
Received 6 May 2016
Received in revised form 22 August 2016
Accepted 27 August 2016
Available online 28 August 2016

Keywords:
Amine group
Nanoparticle
Tumor penetration
Phloretin
PLGA

ABSTRACT

Amine-functionalized poly(lactic-co-glycolic acid) (PLGA-NH_2) nanoparticles (NPs) were developed for the delivery of phloretin. PLGA-NH_2/phloretin NPs with 237 nm mean diameter, narrow size distribution, and around −6 mV zeta potential were fabricated by a modified emulsification-solvent evaporation method. The results of solid state studies revealed that drug was successfully incorporated into the polymeric NPs. The initial particle size of developed NPs was maintained after 24 h incubation in human serum albumin (HSA) solution, fetal bovine serum (FBS), and phosphate buffered saline (PBS). Sustained and higher drug release patterns at acidic pH (pH 5.5), compared with neutral pH (pH 7.4), from PLGA-NH_2 NPs were observed. The experimental data of flow cytometry and confocal laser scanning microscopy (CLSM) studies suggested that PLGA-NH_2 NPs may have an improved cellular accumulation efficiency, compared with PLGA NPs, in Hep-2 cells (human laryngeal carcinoma). Also, PLGA-NH_2 NPs exhibited enhanced growth inhibitory effects rather than PLGA NPs in Hep-2 spheroid model. By introducing a simple strategy based on amine-functionalization of PLGA NPs (without installing complicated functional moieties), improved cellular uptake and antitumor efficacies without severe toxicity, compared with unmodified PLGA NPs, have been accomplished.

1. Introduction

Recently, various types of nanocarriers have been developed for the selective drug delivery to tumor region [1–3]. Macromolecules can be easily extravasated and accumulated in solid tumor due to its leaky vasculature and immature lymphatic system. It is called as an enhanced permeability and retention (EPR) effect and it can be used as a passive tumor targeting strategy [4–6]. However, due to its intrinsic drawback of tumor specificity, other approaches based on ligand and receptor interaction have been introduced as an active tumor targeting strategy [7,8]. For preparing tumor-targetable nanocarriers, a lot of materials have been investigated [9,10]. Biocompatibility and biodegradability of those materials may be requisites for their safe *in vivo* application.

Among them, poly(lactic-co-glycolic acid) (PLGA) has been widely used as one of biodegradable and biocompatible synthetic polymers for the fabrication of nanosystems for anticancer drug delivery [11]. To modulate drug release and improve antitumor efficacy, a lot of polyester derivatives have been synthesized and nanocarriers based on them have been developed [12–14]. In this investigation, amine-functionalized PLGA (PLGA-NH_2) has been introduced to fabricate nanoparticles (NPs) for the delivery of anticancer agents for cancer therapy. The ester linkage of PLGA can be degraded into lactic acid and glycolic acid in the body and they will be eventually eliminated from the body *via* citric acid cycle [15]. However, those acid metabolites can make the adjacent surroundings more acidic [11,16]. Chemical and physical modification of PLGA-based nanocarriers can cover the disadvantages of PLGA and improve the therapeutic potentials [17–19]. PLGA has carboxylic acid groups, thus it can show negative charge after ionization [20]. But, negatively charged NPs may exhibit reduced cellular uptake efficiency due to the repulsion based on their interaction with cellular membrane [21]. Moreover, they can interact with positively charged blood components and make aggregates in the blood stream [21]. Although nanocarriers with cationic surface charge can bind to plasma proteins easily and enter cells efficiently, they can induce higher hematotoxicity [21,22]. Controlling surface charge of nanocarriers can attribute to the optimization of therapeutic potentials. In this study, PLGA-NH_2 NPs have been developed to maximize cellular uptake and tumor penetration efficiency and minimize toxicity.

Phloretin is a dihydrochalcone and it is found in apple tree leaves and the Manchurian apricot. It can inhibit the active transport of glucose into the cells by sodium/glucose cotransporter (SGLT) 1 and

* Corresponding author.
E-mail address: hjcho@kangwon.ac.kr (H.-J. Cho).

http://dx.doi.org/10.1016/j.colsurfb.2016.08.050
0927-7765/

SGLT 2. Higher glycolysis is one of characteristics of solid tumors and it is associated with overexpression of glucose transporters and glycolytic enzymes [23]. Inhibition of glucose uptake into the cancer cells may interfere with their growth, induce apoptosis, and increase their sensitiveness for chemotherapeutics [24–26]. Although the solubility of phloretin has been enhanced by several approaches (*e.g.*, complexation with cyclodextrin and synthesis of its derivatives) [27,28], the delivery systems of phloretin for the therapy of cancers have never been reported, to the best of our knowledge. Its aqueous solubility was known to be 7 μg/mL, thus it should be improved to meet the dose for cancer therapy [28].

Herein, we report about the development and evaluations of PLGA-NH_2/phloretin NPs. Physicochemical properties and *in vitro* stability of NPs, drug release, cytotoxicity, cellular uptake, and the growth inhibition of spheroid were investigated.

2. Materials and methods

2.1. Materials

PLGA (molecular weight: 15–25 kDa, LA:GA = 50:50) and PLGA-NH_2 (molecular weight: 30–40 kDa, LA:GA = 50:50) were bought from PolySciTech (Akina, Inc., West Lafayette, IN, USA). 1,1′-Dioctadecyl- 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), human serum albumin (HSA), and poly(vinyl alcohol) (PVA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Phloretin and sodium dodecyl sulfate (SDS) were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and phosphate buffered saline (PBS) were acquired from Gibco Life Technologies, Inc. (Carlsbad, CA, USA). All other reagents were of analytical grade and obtained from commercial sources.

2.2. Preparation and characterization of phloretin-loaded NPs

PLGA and PLGA-NH_2 were dissolved in chloroform-d ($CDCl_3$) for ^1H NMR analysis (Varian FT–500 MHz; Varian Inc., Palo Alto, CA, USA). The chemical composition of PLGA-NH_2 was measured by X-ray photoelectron spectroscopy (XPS; K-Alpha$^+$, Thermo Fisher Scientific, Waltham, MA, USA). XPS was used for measuring the atomic concentration of C (1s), N (1s), and O (1s) in PLGA-NH_2. Spot size was 30–400 μm (5 μm step).

Phloretin-loaded PLGA NPs and PLGA-NH_2 NPs were prepared using a modified emulsification-solvent evaporation method [12,29]. Briefly, phloretin (6 mg) was dissolved in dimethyl sulfoxide (DMSO; 0.2 mL) and it was mixed with dichloromethane (DCM; 1.3 mL). PLGA or PLGA-NH_2 (60 mg) was solubilized in that solution and mixed with DCM (1.5 mL). That organic phase was added to 15 mL PVA solution (0.5%, w/v) at a rate of 1 mL/min. That emulsion was sonicated for 10 min in the ice using a probe sonicator (VC-750; Sonics & Materials, Inc., Newtown, CT, USA) and the organic solvent was eliminated by stirring for 1 h at room temperature. The aqueous dispersion of drug-loaded NPs was then centrifuged at 13,200 rpm for 30 min and the pellet of NPs was collected by discarding the supernatant. It was resuspended in distilled deionized water (DDW; 3 mL). The centrifugation and resuspension step was repeated three times to remove unencapsulated drug and residual PVA completely. Drug-loaded NPs were lyophilized and stored at −20 °C for further uses.

The mean diameter, polydispersity, and zeta potential of developed NPs were measured using dynamic light scattering (DLS) and laser Doppler methods (ELS-Z1000; Otsuka Electronics, Tokyo, Japan). The encapsulation efficiency (EE) of phloretin in NPs was quantitatively measured using a high-performance liquid chromatography (HPLC) system equipped with a pump (PU-2089 Plus; Jasco, Tokyo, Japan), an automatic injector (AS-2050 Plus), and an UV/Vis detector (UV-1575). NPs were dissolved in DMSO and diluted with mobile phase of HPLC assay. The mobile phase was composed of acetonitrile, DDW, and phosphoric acid (50:50:0.08, volume ratio) and the flow rate was maintained at 1 mL/min. A reverse phase C18 column (Gemini, 250 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) was used and the injection volume was 20 μL. The absorbance of eluent was measured at 288 nm wavelength. The inter- and intra-day variances were within the acceptable range. The morphological shape of phloretin-loaded NPs was observed using a variable pressure-field emission-scanning electron microscope (VP-FE-SEM; SUPRA 55VP, Carl Zeiss, Oberkochen, Germany). NPs were mounted on stubs and coated with Pt under vacuum.

2.3. Solid state studies

Differential scanning calorimetry (DSC) thermograms of phloretin, PLGA/phloretin NPs, and PLGA-NH_2/phloretin NPs were acquired using DSC-Q100 model (TA instruments, New Castle, DE, USA). Aliquot of each sample was scanned in the range of 30–275 °C at 10 °C/min rate under a nitrogen atmosphere.

X-ray diffractometry (XRD) analysis of phloretin, PLGA/phloretin NPs, and PLGA-NH_2/phloretin NPs was conducted with D8 ADVANCE with DAVINCI model (Bruker, Germany). CuKα-radiation (1.5418 Å) was used in 8–45° 2θ range with 0.02 step and 0.5 s/step scan speed at 40 mA and 40 kV of generator conditions.

2.4. In vitro *stability of NPs*

The stability of PLGA/phloretin NPs and PLGA-NH_2/phloretin NPs was evaluated in HSA solution (5%, w/v), FBS (50%, v/v) solution, and PBS (pH 7.4). Freeze-dried NPs were dispersed in each medium at 5 mg/mL and the mean diameter of NPs was monitored by DLS method as described previously. The mean diameters of PLGA/phloretin NPs and PLGA-NH_2/phloretin NPs were measured at 0 h (pre) and 24 h.

2.5. In vitro *drug release*

The release of phloretin from prepared NPs was assessed in PBS media (pH 5.5, 6.8, and 7.4) containing 0.3% (w/v) SDS. Aliquot of NPs dispersion (150 μL), including 200 μg phloretin, was loaded into a mini GeBA-flex dialysis tube (14 kDa molecular weight cut-off; Gene Bio-Application Ltd., Kfar Hanagide, Israel). That dialysis tube was then put into 10 mL PBS (pH 5.5, 6.8, and 7.4 adjusted with phosphoric acid) including 0.3% (w/v) SDS. Those samples were incubated in a shaking bath at 37 °C with 100 rpm agitation speed. Aliquots (200 μL) of release media were collected at determined times (2, 4, 6, 24, 48, 72, 96, and 168 h) and an equivalent volume of fresh media was supplemented. The released amounts of phloretin in the release media were quantitatively determined by described HPLC method. The stability of phloretin in each release medium (pH 5.5, 6.8, and 7.4 buffers including 0.3% SDS) was also measured by described HPLC method. Excess amount of phloretin was added to each medium and vortex-mixed. That sample was centrifuged at 13,200 rpm for 5 min and the supernatant was filtered through syringe membrane filter with 0.2 μm pore size. After incubating at 37 °C for 168 h, the drug concentration was determined and the degradation degree was calculated.

2.6. In vitro *cytotoxicity*

Hep-2 cells were acquired from the Korean Cell Line Bank (Seoul, Korea). Hep-2 cells were cultured in DMEM including 10% (v/v)