Biomaterials 71 (2015) 71–83

Contents lists available at ScienceDirect

Biomaterials

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

Tumortropic monocyte-mediated delivery of echogenic polymer bubbles and therapeutic vesicles for chemotherapy of tumor hypoxia

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ARTICLE INFO

Article history:
Received 17 March 2015
Received in revised form
14 August 2015
Accepted 16 August 2015
Available online 19 August 2015

Keywords:
Tumortropic monocytes/macrophages
Tumor hypoxia
Cell-mediated drug delivery
Co-delivery

ABSTRACT

Overcoming limitations often experienced in nanomedicine delivery toward hypoxia regions of malignant tumors remains a great challenge. In this study, a promising modality for active hypoxia drug delivery was developed by adopting tumortropic monocytes/macrophages as a cellular vehicle for co-delivery of echogenic polymer/C_5F_{12} bubbles and doxorubicin-loaded polymer vesicles. Through the remote-controlled focused ultrasound (FUS)-triggered drug liberation, therapeutic monocytes show prominent capability of inducing apoptosis of cancer cells. The *in vivo* and *ex vivo* fluorescence imaging shows appreciable accumulation of cell-mediated therapeutics in tumor as compared to the nanoparticle counterpart residing mostly in liver. Inhibition of tumor recurrence with γ-ray pre-irradiated Tramp-C1-bearing mice receiving therapeutic monocytes intravenously alongside the FUS activation at tumor site was significantly observed. Immunohistochemical examination of tumor sections confirms successful cellular transport of therapeutic payloads to hypoxic regions and pronounced cytotoxic action against hypoxic cells. Following the intravenous administration, the cellular-mediated therapeutics can penetrate easily to a depth beyond 150 μm from the nearest blood vessels within pre-irradiated tumor while nanoparticles are severely limited to a depth of ca 10–15 μm. This work demonstrates the great promise of cellular delivery to carry therapeutic payloads for improving chemotherapy in hypoxia by combining external trigger for drug release.

1. Introduction

Over the past two decades, nanoparticle (NP)-based delivery systems have shown great potential in selectively transporting therapeutic and diagnostic agents to tumor sites by either passive targeting or combined passive and active targeting [1,2]. Passive targeting is generally achieved via the enhanced permeability and retention (EPR) effect as long as the capture of NPs by the reticuloendothelial system occurring primarily in liver and spleen can be largely circumvented. Once therapeutic NPs arrive at tumor sites, the selectivity to target cells can be enhanced by implementation of homing ligands such as short peptides, antibodies and nucleotide-based aptamers on NP surfaces via highly specific binding with cell membrane receptors [3]. Despite of remarkable progress in the development of multifunctional nanoscale vehicles for improved cancer therapy, the greatly retarded penetration of therapeutic payloads into deep extravascular tumor tissue is one of the main issues that limit the therapeutic efficacy for solid tumors.

The tumor microenvironment is rather heterogeneous. For instance, malignant solid tumors are composed of a variety of cells including cancer cells, monocytes and monocyte-derived tumor associated macrophages, lymphocytes, fibroblasts and stem cells, etc [4]. The compact and dense extracellular matrix, high interstitial fluid pressure and irregular blood supply found in tumor tissue often serve as physical barriers to inhibit penetration of chemo- or radiotherapeutic agents across tumor blood microvessels into tumor avascular cores [4–6]. Being spatially adjacent to tumor central necrotic areas, hypoxia regions are rather distant from the nearest

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http://dx.doi.org/10.1016/j.biomaterials.2015.08.033
0142-9612/

blood microvessels (ca 70–100 μm) within human tumor and thus featured with insufficient levels of oxygen and glucose for the basic metabolic requirement of tumor cells therein. Nevertheless, several reports suggest that tumor cells in hypoxic regions are closely associated with early biochemical relapse after radiotherapy and thus with local recurrence and distant metastasis [7–9]. Unfortunately, because of the void of blood microvessels, which leads to the deficiency of oxygen required by radiation treatment to generate reactive species and the limitation in diffusion of chemotherapies into deep interior hypoxia, these regions are considered to be highly resistant to chemo- and radiotherapy [10,11]. There are also increasing evidences showing that nano-scale delivery systems are virtually too large in size (ca 100 nm) to extravasate deep into tumor regions (limited to a penetration depth of only 1–2 cell layers from tumor neovessels) [12–14].

Tumor hypoxic cells secrete varying chemokines and factors to promote migration of tumortropic non-malignant cells and their attachment to tumor hypoxic areas [15,16]. In this regard, employing bone marrow-derived monocytes/macrophages to serve as a delivery vehicle for improving the drug distribution in tumors, particularly accumulation in hypoxia regions, was proposed recently [17–21]. It is crucial that the cellular host retains not only viability but also migratory selectivity toward tumor hypoxia, while therapeutic payloads can be activated for effective cancer therapy by external trigger upon arriving at the target sites. In this study, the bone marrow-derived monocytes/macrophages isolated from C57BL/6J mice were employed as the cellular Trojan horse simultaneously carrying therapeutic polymersomes and echogenic polymer bubbles for externally triggered chemotherapy of tumor hypoxia. Focused ultrasound (FUS) was chosen to serve as the external acoustic trigger by inducing membrane damage of doxorubicin (DOX)-loaded polymer vesicles (DLPV) from inertial bubble cavitation for controlled drug release. Characterizations of cell-mediated therapeutics with respect to NP payload uptake, ultrasound (US) imaging, FUS-mediated intercellular drug transport from host to tumor cells and cytotoxic action against cancer cells, irradiated tumor medium-stimulated monocytic migration and tumortropism were performed. We also employed Tramp-C1-bearing mice (C57BL/6J) as an animal model for evaluating *in vivo* chemotactic recruitment of therapeutic monocytes and chemotherapy efficacy, involving the use of multiple immunohistochemical (IHC) staining of tumor tissue sections. The tumor was pretreated with γ-ray irradiation to promote the hypoxia formation for better evaluating hypoxia selectivity of the cellular Trojan system. Fig. 1 illustrates a schematic strategy adopted in this work for co-delivery of echogenic polymer bubbles and therapeutic polymersomes to tumor hypoxia via tumortropic monocytes for FUS-triggered chemotherapy.

2. Materials and methods

2.1. Materials

Poly(acrylic acid-co-distearin acrylate) (poly(AAc-co-DSA)) of two different DSA contents (ca 15 and 25 mol%, denoted hereinafter as PAAc-d15 and PAAc-d25) was prepared and characterized according to the methods described previously [22–24]. The synthetic route is schematically depicted in Fig. S1 (supplementary data). The synthesis recipe, composition and average molecular weight of these copolymers are summarized in Table 1. Tramp-C1 mouse prostate cancer cell line was purchased from American Type Culture Collection (CRL-2730), and mouse macrophage-like RAW264.7 cell line was from Food Industry Research and Development Institute (Hsinchu, Taiwan). Tramp-C1 and RAW 264.7 cells were both maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Long Island, NY) with 10% FBS (HyClone) and 1% penicillin-streptomycin (Gibco). Cell lines were incubated at 37 °C in humidified 5% CO_2/air atmosphere [25]. Six-to 8-week-old C57BL/6JNarl male mice were purchased from National Laboratory Animal Center, Taiwan. The approved guides for the care and use of laboratory animals by the Institutional Animal Care and Use Committee (IACUC) of National Tsing Hua University, Taiwan (approved number: IACUC:10129) were followed at all time. All surgeries were performed under Zoletil/Rompun anesthesia, and all efforts were made to minimize suffering.

2.2. Preparation of polymer bubbles (PB) and DLPV

PB were prepared by the emulsion/nanoprecipitation technique. PAAc-d25 (3.0 mg) was first dissolved in the solution (1.0 mL) of THF and perfluoropentane (C_5F_{12}) at a volume ratio of 85/15. Phosphate buffer (*I* 0.01 M, pH 7.4, 2.0 mL) was vigorously mixed with the polymer solution by an ultrasonic homogenizer (Cole-Parmer, VCX 750) at 750 Watt in ice-water bath for 10 min. The resultant emulsion was continuously stirred by purging nitrogen for 2 h to remove THF and untrapped C_5F_{12}. This emulsion was then dialyzed against phosphate buffer (*I* 0.01 M, pH 7.4) for 24 h at 4 °C to completely remove organic solvents. The resultant polymer droplet suspension was incubated at 37 °C in a rotary shaker at 100 rpm. After 24 h incubation, PB was attained. Schematic illustration of the preparation and echogenic properties of the C_5F_{12}-entrapped PB are shown in Fig. S2.

DLPV was prepared by the nanoprecipitation technique. PAAc-d15 (5.0 mg) was dissolved in THF (1.0 mL). The copolymer solution (0.5 mL) was added dropwise into acetate buffer (pH 5.0, 0.5 mL, *I* 0.01 M) containing DOX (Seedchem, Australia) at a concentration of 1.5 mg/mL. The mixture was then subjected to ultrasonic dispersion (Powersonic 410) for 5 min at 40 °C, followed by equilibration at 25 °C for 30 min. After dialysis (Cellu Sep MWCO 6000–8000) against pH 5.0 acetate buffer to remove THF and unloaded DOX species, DLPV was obtained. To assess the drug loading level, an aliquot of DLPV was withdrawn and diluted and disintegrated with DMF to a volume ratio of DMF/H_2O = 9/1. The amount of DOX encapsulated was determined by a fluorescence spectrophotometer (Hitachi F-7000, Japan). The excitation was performed at 480 nm and the emission spectrum was recorded in the range of 500–700 nm. Drug loading efficiency (DLE) and drug loading content (DLC) were calculated according to the following formulas, respectively:

$$\text{DLE}\ (\%) = \frac{\text{weight of loaded DOX}}{\text{weight of DOX in feed}} \times 100\,\%$$

$$\text{DLC}\ (\%) = \frac{\text{weight of loaded DOX}}{\text{weight of copolymer in feed}} \times 100\,\%$$

Drug release from DLPV was characterized by a dialysis method. The released DOX concentration was determined by fluorescence measurements using two calibration curves of DOX established in aqueous solutions of pH 5.0 and 7.4, respectively.

2.3. Structural characterizations

The mean hydrodynamic diameter (D_h) and size distribution (polydispersity index, PDI) of polymer assemblies (PB and DLPV) in aqueous solution were determined by dynamic light scattering (DLS) on a Brookhaven BI-200SM goniometer equipped with a BI-9000 AT digital correlator using a solid-state laser (30 mW, λ = 637 nm) at 90°, based on the Cumulant method. The results reported herein represent an average of at least triplicate measurements.