Biomaterials 145 (2017) 56–71

Contents lists available at ScienceDirect

Biomaterials

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

Co-delivery of oxygen and erlotinib by aptamer-modified liposomal complexes to reverse hypoxia-induced drug resistance in lung cancer

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ARTICLE INFO

Article history:
Received 28 May 2017
Received in revised form
13 August 2017
Accepted 15 August 2017
Available online 18 August 2017

Keywords:
Liposomal complexes
Hypoxia
Drug resistance
PFOB
NSCLC

ABSTRACT

Tumor hypoxia is a common feature of the tumor microenvironment and has been regarded as one of the key factors in driving the emergence of drug resistance in solid tumors. To surmount the hypoxia-associated drug resistance, we fabricated the novel multifunctional liposomal complexes (ACLEP) that could co-deliver oxygen and molecular targeted drug to overcome the hypoxia-induced drug resistance in lung cancer. The ACLEP were fabricated with liposomes anchored with *anti*-EGFR aptamer-conjugated chitosan to co-administrate erlotinib and PFOB to EGFR-overexpressing non-small-cell lung cancer. Our results showed that the ACLEP possessed desired physicochemistry, good biostability and controlled drug release. The entrapped PFOB in nanoparticle facilitated the uptake of ACLEP in either normoxia or hypoxic condition. Comparing to those nanoparticles loading erlotinib alone, our innovative oxygen/therapeutic co-delivery system showed a promising outcome in fighting against hypoxia-evoked erotinib resistance both *in vitro* and *in vivo*. Hence, this work presents a potent drug delivery platform to overcome hypoxia-induced chemotherapy resistance.

1. Introduction

Lung cancer, more than 85% of which are classified as non-small cell lung cancer (NSCLC), is one of the leading causes of cancer mortality and is responsible for about 25% of cancer death [1,2]. Epidermal growth factor receptor (EGFR) is a highly expressed protein in NSCLC cells, and the activation of it is keenly associated with tumor cell cycle progression, invasion, metastasis, angiogenesis, and apoptosis [3–5]. EGFR-tyrosine kinase inhibitors (EGFR-TKIs) have emerged in treating NSCLC, by reversibly and selectively inhibiting EGFR expression [6,7]. Unfortunately, the emergence of drug resistance to these EGFR-TKIs is inevitable in patients after a medication of approximately one year [8]. The most investigated mechanisms for EGFR-TKIs resistance include the secondary mutations and tyrosine kinase switches [9]. However, the mechanisms responsible for intrinsic and acquired resistance to EGFR-TKIs are not only the cellular factors which are related to functional gene mutations and changed survival/apoptotic pathways [8], but also the physiological factors involving tumor microenvironment and tumor physiology [10–12]. As all the factors are inter-related and mutually dependent, targeting the physiological factors could also be an effective way to reverse EGFR-TKIs resistance [12].

Hypoxia, defined as the inadequate oxygen supply at the tissue level, is a common pathological condition in tumor microenvironment of the majority of solid tumors, leading to the restricted drug influx and the development of drug resistance [8,13,14]. Hypoxia-inducible factor-1α (HIF-1α), an oxygen regulated subunit of HIF-1 [15], could be responsively up-regulated when tumor cells are exposed to low oxygen concentration, triggering the promotion of new blood vessel formation and acceleration of tumor growth [8,16]. Previous studies have reported that hypoxia up-regulated HIF-1α expression and remarkably increased the population of cancer cells resistant to EGFR-TKIs in NSCLC [17,18]. In view of the close association between hypoxia and EGFR-TKIs resistance [18], an effective hypoxia-targeting strategy is exigently needed to ameliorate the hypoxic tumor microenvironment and reverse

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http://dx.doi.org/10.1016/j.biomaterials.2017.08.030
0142-9612/

EGFR-TKIs resistance in NSCLC.

Hemoglobin and perfluorocarbon (PFC) are two main classes of artificial blood substitutes for supplying sufficient oxygen to the body [19,20]. Compared to hemoglobin, PFC is capable of dissolving more amounts of oxygen and the soluble oxygen released from PFC can simply diffuse along the concentration gradient with high extraction ratios [15,21]. The hypotoxic, nonvolatile and chemically stable properties of PFC make it possible to be used in numerous biomedical and bioprocess systems [22–26]. Perfluorooctylbromide (PFOB) is a type of PFC [27], which has been studied extensively for its good diffusibility, low surface tension, low viscosity, high density, and high gas solubility [28]. Therefore, to relieve hypoxia-triggered drug resistance, PFOB is a good choice to supply oxygen for hypoxic tumor tissue.

To improve therapeutic efficacy, the therapeutic substances should be effectively delivered to the target sites. However, the insufficient oxygen availability and vascular blood flow in hypoxic tumor tissues resulted in poor drug delivery [29]. Nanoparticulate drug delivery system (NDDS) has great potential to significantly and specifically enhance drug accumulation in hypoxic tumor cells [4,30]. Especially the targeted nanoparticulate drug delivery system (TNDDS), of which the surface was modified with ligands, have demonstrated better therapeutic outcomes, by propelling the loaded drugs into tumor cells through receptor-mediated internalization [31]. In addition, NDDS provides a promising platform for combination therapy [32] which is a widely employed strategy in clinic to combat drug resistance [33]. Currently, some articles have reported using microbubbles as effective oxygen-delivery vehicles to relieve the tumor hypoxia and improve sonodynamic therapeutic effect [34,35]. In addition to being used as a contrast agent for ultrasound imaging, oxygen carrier could also be explored as an artificial blood substitute against hypoxia-induced drug resistance [15]. Therefore, it would be interesting to develop nanotherapeutics containing oxygen and EGFR-TKIs synchronously, which could modulate hypoxic tumor microenvironment to improve therapeutic outcomes. In this work, to modulate EGFR-TKI resistance induced by hypoxia in lung cancer treatment, both PFOB and EGFR-TKI (erlotinib) were packaged in one nano-carrier, thus acting as an efficient NDDS to achieve therapeutic synergy. Furthermore, biodegradable NDDS has ability to improve the solubility/bioavailability of its entrapped poorly soluble drugs [36]. Because of the poor water-solubility of PFOB and erlotinib, it is necessary to choose suitable nanomaterials to help them maintain in water. Liposome, owing to the structure of bilayer with a hydrophilic core and hydrophobic shell, has attracted keen interest to circumvent this problem [37]. In prior works, we have revealed that chitosan (Cs)-anchored liposomal complexes (CL) could perform as an excellent NDDS with superior stability than conventional unmodified ones [38]. Therefore, in this work, CL were selected as nano-carriers to administrate drug and PFOB together.

The ideal targeting agents should have a high affinity to receptors on diseased or activated cells, whereas have a low affinity to receptors expressed in normal cells [39]. Nucleic acid aptamers (Apts) as target molecules offer unprecedented opportunities for effective receptor mediation, permitting specific penetration into biological compartments with non-immunogenicity [40–43]. Additionally, their inherent anti-tumor efficacy is also favored for Apt-modified nanoparticle platforms [44]. Thus, EGFR-specific Apt could serve as a promising targeting agent for specific recognization and regulation of EGFR-overexpressing lung cancer cells. However, there is still no literature available now concerning targeted drug and oxygen co-delivery system to overcome the hypoxia-induced drug resistance in the EGFR-activated NSCLC therapy.

Herein, we formulated novel multifunctional liposomal complexes, ACLEP, in which *anti*-EGFR Apt-conjugated chitosan (Apt-Cs) was anchored into liposomes to co-administrate erlotinib and PFOB for reversing hypoxia-induced drug resistance. The physicochemical characteristics of the multifunctional liposomal complexes were first determined by evaluating its size, polydispersity index (PDI), zeta potential, morphology, stability, oxygen content, and drug release profiles. Then the cellular uptake efficiency, cell viability, cell apoptosis, and the related protein expression mediated by ACLEP were investigated in different NSCLC cells under normoxic condition and hypoxic condition. Finally, *in vivo* experiments were conducted in NSCLC-bearing mouse model to examine the feasibility of using ACLEP to lessen drug resistance.

2. Experimental section

2.1. Materials

Lecithin, cholesterol, and Cs were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Erlotinib was purchased from Mellon, Biological Product Co., Ltd. (Dalian, China). 1-Bromoheptadecafluorooctance (PFOB) was provided by Aladdin Industrial Corporation. 1-Ethyl-3-(3- dimethly-aminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were bought from Aladdin Reagent Co., Ltd. (Shanghai, China). 4′,6- diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342 was purchased from Beyotime (Shanghai, China). Trypsin-EDTA, penicillin/streptomycin, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were obtained from Gibco-BRL (Burlington, ON, Canada). The RPMI 1640 medium and dulbecco's modified eagle medium (DMEM) were purchased from Life Technologies GmbH (Darmstadt, Germany). The rabbit anti-human EGFR, *p*-EGFR, HIF-1α, GAPDH antibodies were procured from Wanleibio Co., Ltd. (Shenyang, China). The secondary antibody goat anti-rabbit IgG horseradish peroxidase conjugate was obtained from Promega Co., Ltd. (Shanghai, China). All the other reagents used in this study were of analytical grade.

The high performance liquid chromatography (HPLC)-purified *anti*-EGFR DNA aptamer (5′-carboxyl-TGA ATG TTG TTT TTT CTC TTT TCT ATA GTA -3′) with or without 3′- fluorescein isothiocyanate (FITC) modification was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequence of the Apt was reported in previous research [45].

2.2. Synthesis of Apt-Cs

To formulate targeted nanoparticles, *anti*-EGFR Apt was covalently conjugated to the Cs backbone through cross-linking reagents EDC and NHS. Briefly, 10 mg EDC and 8 mg NHS were dissolved in 2 mL distilled water followed by the addition of 100 μL of Apt solution (10 μM). The mixture was then stirred to activate the carboxylic group at room temperature for 3 h under light-sealed condition. Subsequently, 200 μL of Cs (1% w/v) solution was added to the above solution, and stirred overnight. The excessive EDC, NHS, and Apt were removed by ultrafiltration centrifugation (MWCO = 10 kD) to obtain purified Apt-Cs. The mother liquor was diluted with PBS (pH = 7.4) for further investigation.

2.3. Preparation of ACLEP

Liposomes were prepared according to the previous literature [46]. Based on this method, CL or Apt-Cs anchored liposomal complexes (ACL) were formulated by substituting Cs or Apt-Cs