Materials Science & Engineering C 89 (2018) 371–377

Contents lists available at ScienceDirect

Materials Science & Engineering C

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

The effect of the androstane lung cancer inhibitor content on the cell-selective toxicity of hydroxyapatite-chitosan-PLGA nanocomposites

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ARTICLE INFO

Keywords:
Androstane
Hydroxyapatite
Nano-carrier
Lung cancer
Cell-selective cytotoxicity

ABSTRACT

An androstane (17β-hydroxy-17α-picolyl-androst-5-en-3β-yl-acetate (derivative A)) cancer inhibitor was successfully captured in a carrier made of nano-sized hydroxyapatite (HAp) coated with chitosan-PLGA polymer blends (Ch-PLGA). In our previous studies, we demonstrated that it was convenient to use spherical HAp/Ch-PLGA carriers as vehicles to target the lungs following intravenous administration. In this study, we used emulsification and subsequent freeze-drying to load the spherical HAp/Ch-PLGA carriers with varying contents of the derivative A, in order to examine the selective toxicity towards cancerous/healthy lung cells. The XRD and FT-IR techniques confirmed the drug loading process, and the content of the poorly water soluble derivative A was estimated directly *via* the DSC technique. The particles were spherical in shape with the d_{50} distribution varying between 167 and 231 nm, whereas the content of the derivative A ranged from 6.5 to 19.3 wt%. Cell-selective cytotoxicity was examined simultaneously on two cell lines: human lung carcinoma (A549 ATCC CCL 185) and human lung fibroblasts (MRC-5 ATCC CCL 171). All particles exhibited nearly three times larger cytotoxicity towards cancer cells (A549) than towards healthy cells (MRC5), where the particles with the derivative A content of 6.5 wt% allowed for the viability of healthy cells > 80%. Ninety-six hours after the treatment of cells with particles with different contents of derivative A (after incubation and recovery), recovery was faster in damaged healthy cells than in cancerous cells.

1. Introduction

Calcium phosphates, especially hydroxyapatite (HAp), as the most common inorganic bone component [1], have a high potential for use in preventive and regenerative medicine [2,3]. The past decade has witnesses a significant growth of research into the application of HAp particles, not only as a material for the reconstruction of bone tissue but also as a drug carrier in biomedicine [4]. Hydroxyapatite nanoparticles (HApNs) have shown selective anti-cancer activity in the treatment of lung cancer. The cytotoxic activity of HApNs towards human lung cancer cells (A549) has been demonstrated to be significant, without affecting the survival of normal bronchial epithelial 16HBE cells [5]. The research aimed at creating new functional and hybrid systems based on HAp largely expands the potential for its application for biomedical purposes [6]. In our previous study, we examined the possibility of applying multifunctional and hybrid systems based on HAp and bioresorbable polymers in the reconstruction of bone tissue damaged by osteoporosis [7,8].

Nano-carriers for cancer inhibitors are a promising strategy in increasing the treatment efficiency for various types of cancer, accompanied with an increased toxicity towards cancer cells and decreased toxicity towards healthy cells [9]. Lung cancer is one of the most common and the most lethal cancer types at a global level [10]. An analysis of the delivery efficiency of nanoparticles in different tumor types indicates that it is the lowest in cases of lung tumors [11]. There are different strategies seeking not only to improve the delivery efficiency, but also to increase the drug content in the carrier. One of them is to design a synthetic system where the system/carrier molecules would firmly grip and entrap the drug. These cage nanoparticles could be promising vehicles for continuous drug delivery [12].

Biodegradable polymer particles based on poly-lactide-*co*-glycolide (PLGA) have been used as suitable vehicles for various cancer inhibitors targeting the lungs after intravenous application [13]. Core-shell particles based on PLGA and linear polysaccharide chitosan (Ch) loaded

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https://doi.org/10.1016/j.msec.2018.04.028
Received 27 November 2017; Received in revised form 4 April 2018; Accepted 12 April 2018
Available online 14 April 2018
0928-4931/

with cancer inhibitors have shown a high potential in the treatment of lung cancer [14,15]. PLGA nanoparticles modified with Ch have also shown a high potential as nano-carriers of poorly hydrophilic drugs in the treatment of breast cancer [16]. Dual-drug containing core–shell nanoparticles based on PLGA and Ch were designed to reduce and eliminate toxicity to healthy human cells in potential lung cancer therapy and their efficiency was tested. Particles with the hydrodynamic diameter of 289 ± 49 nm were cyto- and chemo-compatible, and they operated by targeting folate receptors [17]. During *in vivo* studies, it was found that after the intravenous application of spherical nano-HAp-based particles coated with a polymeric PLGA-Ch blend, they were accumulated in the lungs [18,19]. Spherical HAp/Ch-PLGA particles with hydrodynamic properties that allow to direct them towards the lungs following injection could play the role of vehicles in drug delivery for various lung diseases. Polymer components that coat nano-HAp particles could be optimal for the entrapment of various drugs. A wide variety of different androstane derivatives with a heterocyclic ring showed a high potential as cancer inhibitors in the treatment of hormone-dependent cancers [20–22].

In our previous research, we examined the possibility of synthesizing and designing HAp/Ch-PLGA spherical particles loaded with17β-hydroxy-17α-picolyl-androst-5-en-3β-yl-acetate (A). The results of *in vitro* tests indicated selective A-HAp/Ch-PLGA activity; the particles did not exhibit cytotoxicity towards primary mouse lung fibroblasts (C57BL/6) but they were cytotoxic for lung cancer cells [23]. In the present study, we demonstrate the synthesis and design of spherical HAp/Ch-PLGA particles loaded with varying contents of an androstane-based cancer inhibitor (17β-hydroxy-17α-picolyl-androst-5-en-3β-yl-acetate, A). The influence of the content of androstane derivative on the morphology and size distribution of the synthesized particles was also examined. The content of androstane derivatives in all systems was determined directly using the DSC technique. The electrokinetic parameters of all particles as potential activity indicator in the middle particle layer – biological environment were determined by measuring zeta potential, electrophoretic mobility and conductivity. The influence of the content of the androstane-based cancer inhibitor A on cell-selective toxicity was examined simultaneously on two cell lines: on human lung carcinoma (A549) and human lung fibroblasts (MRC-5) using dye exclusion (DET) and MTT assays. The viability of both cell types after the treatment with synthesized particles was also examined in order to quantify the selective activity.

2. Experimental

2.1. Synthesis of materials

2.1.1. Synthesis of 17β-hydroxy-17α-picolyl-androst-5-en-3β-yl acetate (A)

The first stage in the preparation of 17β-hydroxy-17α-picolyl-androst-5-en-3β-yl-acetate (A) was the addition of α-picolyllithium to the 17-oxo group of dehydroepiandrosterone, resulting in 17α-picolyl-androst-5-en-3β,17β-diol. This was followed by acetylation using acetic anhydride in absolute pyridine at room temperature for 3 h [24,25].

2.1.2. Synthesis of HAp, HAp/Ch-PLGA and A-loaded HAp/Ch-PLGA

An aqueous calcium nitrate ($Ca(NO_3)_2$) solution (150 ml; 26.6 wt%) was added to the solution of ammonium phosphate ($(NH_4)_3PO_4$) (7 ml H_3PO_4 + 165 ml NH_4OH + 228 ml H_2O) at 50 °C over the period of 60 min, while stirring at the rate of 100 rpm. The solution was then subjected to a heat treatment at 100 °C for 60 min. The resulting gel was dried at room temperature in a vacuum drier for 72 h, after which the final product – HAp powder - was obtained. X-ray diffraction run on the HAp powder confirmed its poorly crystalline nature, whereas the PSD technique enabled us to determine the particle size of d_{50} = 70 nm [26].

Chitosan (Ch) of a low molecular weight (Aldrich, deacetylation > 75%), dissolved in acetic acid (1 wt%), was mixed with 17β-hydroxy-17α-picolyl-androst-5-en-3β-yl acetate powder (A) in the weight ratio according to Table 1, while stirring with a magnetic stirrer at 400 rpm. PLGA (50:50, Sigma, USA) dissolved in acetone was mixed with the A-containing Ch solution and HAp gel in the 2:3:5 weight ratio. A water solution of poloxamer 188 (polyethylenepolypropylene glycol, 0.1 vol%) was added drop-wise to the resulting mixture, while stirring at 21,000 rpm. The obtained mixture of A, chitosan, PLGA and HAp was slowly poured into a glutaraldehyde solution (Grade I, 25% in H_2O), while stirring at 21,000 rpm for 1 h. The obtained mixture was then centrifuged at 3000 rpm and 5 °C for 1 h, and the resulting precipitate was subjected to lyophilization at temperatures ranging from −10 to −60 °C and pressures ranging from 0.37 mbar to 0.1 mbar for 1 to 8 h [23]. The obtained powder was washed with distilled water three times, centrifuged at 1000 rpm and dried again. The final product was the powder composed of HAp particles coated with A-loaded chitosan-poly(D,L)-lactide-*co*-glycolide (Table 1).

Table 1
The projected content of A in A-loaded HAp/Ch-PLGA.

A content [wt%]	Name
10	A_1-HAp/Ch-PLGA
15	A_2-HAp/Ch-PLGA
25	A_3-HAp/Ch-PLGA

2.2. Characterization of the products

X-Ray diffraction (XRD) was performed on a Philips PW-1050 diffractometer with Ni-filtered $Cu_{K\alpha}$ radiation, the scanning step was 0.02°. The particle size distribution (PSD) was measured on 10 mg/ml of powders dispersed in water using a Mastersizer 2000 (Malvern Instruments Ltd.) and a *HydroS* dispersion unit for liquid dispersants. Morphological characterization was performed using an atomic force microscope (AFM, Thermo Microscopes, Autoprobe CP Research). Content of HAp in the particles was determined by simultaneous TG − DTA (Setsys 2400 CS Evolution, SETARAM Instrumentation, Caluire, France) in the temperature range between 30 and 600 °C with the heating rate of 10 °min^{-1} and under the air flow of 20 ml min^{-1}. Differential scanning calorimetry (DSC) measurements were performed on an Evo 131 (Setaram Instrumentation) differential scanning calorimeter. Samples were analyzed in nitrogen by heating (10°/min) from 25 to 360 °C. Infrared spectroscopy (FT-IR) was done on a Nicolet iS10 FT-IR Spectrometer (Thermo Scientific Instruments) in the spectral range from 400 to 4000 cm^{-1}. Electrokinetic parameters of the suspensions of synthesized particles were analyzed using a Zeta-Sizer Nano (Malvern Instruments Ltd.) in deionized water and pH 7.0.

2.3. In vitro tests

2.3.1. Cell lines

Toxicity tests were carried out on two cell lines, human lung carcinoma (A549 ATCC CCL 185) and human lung fibroblasts (MRC-5 ATCC CCL 171) which grew adhered to the dish floor (Costar, 25 cm^3) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, UK) with 4.5 g/l glucose and 10% FCS (fetal calf serum, Sigma). The medium contained antibiotics: penicillin 100 IU/ml and streptomycin 100 μg/ml. The cell lines were maintained under standard conditions: at the temperature of 37 °C in a humidity saturated atmosphere with 5% CO_2 (Heraeus). The cells were passaged twice a week, and in the experiments the cells were used in the logarithmic growth phase between the third and tenth passages. Only viable cells were used. The number of cells and their viability were determined by a color rejection test with 0.1% trypan blue.