Photodiagnosis and Photodynamic Therapy 21 (2018) 138–146

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

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

Synthesis of calcium phosphate nanostructures by combustion in solution as a potential encapsulant system of drugs with photodynamic properties for the treatment of cutaneous leishmaniasis

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ARTICLE INFO

Keywords:
Calcium phosphates
Nanostructures
Encapsulation
Hypericin
Photodynamic therapy
Cutaneous leishmanisis

ABSTRACT

The traditional drugs used in the treatment of cutaneous leishmanisis (CL) have multiple disadvantages, such as high toxicity, high costs, and more recently the appearance of parasites resistant to those drugs. For this reason, some research has focused on the development of new drugs or treatment therapies. Photodynamic therapy (PDT) that involves the use of a photosensitive or photosensitizing compound capable of producing reactive oxygen species to which Leishmania parasites are sensitive, has emerged as an alternative for the treatment of CL. However, some of these sensitizing compounds exhibit some toxicity (cytotoxicity, allergic reaction, etc), low selectivity, and some of them are insoluble in aqueous media limiting their use. Therefore, the PDT can be improved using encapsulation systems which protect drugs, prevent their degradation, help them overcome physical barriers and increase their selectivity. In this study, we propose the use of calcium phosphate as a potential encapsulant or photodynamic support for photoactive drugs, using hypericin (HY) as a photosensitizer agent. The self-combustion route was used to synthesize the CP nanostructures. The structure and morphology of CP nanoparticles were evaluated via X-ray diffraction (XRD), Raman and field-emission scanning electron microscopy (FE-SEM). Phases rich in hydroxyapatite and CP β phase, with granular morphology and an average grain size of 42.9 nm were obtained. The encapsulation uptake and the interactions between HY and the encapsulated system were evaluated by fluorescence spectroscopy and Fourier-transform infrared spectroscopy (FTIR), respectively. Approximately 13% of HY was enapsulated per 1 μg of nanoparticles of calcium phosphate. Composites were submitted to *in vitro* assays of cytotoxicity and anti-leishmanial activity. The CP nanoparticles did not affect the photodynamic activity of HY. On the contrary they showed antileishmanial response.

1. Introduction

The calcium phosphate [1] is a ceramic material with broad biomedical and biological applications due to its excellent biocompatibility. The use of CP has been extended from the manufacture of implants for bone regeneration to implementation as encapsulation systems for the controlled loading and release of drugs [2,3]. The CP such as hydroxyapatite have applications in gene therapy and incorporation of anticancer drugs, and once doped, it is possible to extend its application to sunscreens, drug release through localized therapy, hyperthermia, etc. [4–7]. Currently, CP is being applied in tissue engineering and in the management of diseases considered of prime interest in the pharmaceutical industry as cancer [8]. However, the potential use of CP in neglected tropical diseases as leishmaniasis has not been explored.

Leishmaniasis is a parasitic disease caused by protozoans of the genus *Leishmania*, which are transmitted by the bite of vector insects of the genera *Phlebotomus* and *Lutzomia*. There are three main clinical forms: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucosal leishmaniasis (ML) being CL the most predominant form [9,10]. The disease is present in 98 countries worldwide and mainly affects the economically most vulnerable populations. It is estimated that approximately 350 million people are at risk of get infected and between 0.7 and 1.3 million new cases of cutaneous leishmaniasis (CL) occur every year [11]. In Colombia, 10561 cases of leishmaniasis were reported in 2016. Only four therapeutic alternatives are available to treat leishmaniasis: pentavalent antimonial (meglumine antimoniate or sodium stibogluconate), miltefosine, pentamidine isethionate, and amphotericin B. In general, these drugs are highly toxic, with important adverse effects and they are expensive. In addition, strains resistant to

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https://doi.org/10.1016/j.pdpdt.2017.11.017
Received 24 June 2017; Received in revised form 28 November 2017; Accepted 29 November 2017
Available online 02 December 2017
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some of these drugs have been reported making them far from being the ideal drugs for the treatment of the disease [9,12–14]. It is therefore imperative to develop new drugs and/or therapeutic alternatives for the treatment of leishmaniasis. Today, the efficacy of different therapies such as thermotherapy, cryotherapy [11] and photodynamic therapy (PDT) are being explored [15–17].

The PDT consists of the administration of a photosensitizer agent that accumulates in the desired area. Once exposed to a light source, with an appropriate wavelength, reactive oxygen species (ROS) are release and, oxidization of lipids, carbohydrates, and proteins occurs, promoting thus the elimination of the parasite [18]. Different photosensitizers have exhibited anti-leishmania activity; this is the case of methylene blue [19,20], 3,7-bis (N, N-dibutylamino) phe-nothiazinium bromide (PPA904) [21,22], D-aminolevulinic acid-derived protoporphyrin IX (ALA) [[18], chloro aluminum phthalocyanine, free or encapsulated in ultra-deformable liposomes (UDL-ClAlPc) [23,24], dimethyl and diethyl carbaporphyrin ketals [25,26], zinc phthalocyanine suspended in TiO_2 [27], zinc phthalocyanine liposomes [28], metvix [29], riboflavin [30], silver-doped inorganic compounds such as Ag-Nanoparticles (NPs) [31], liposomated zinc oxide[32], TiO_2 doped with Ag [33], Au-NPs, TiO_2 NPs, ZnO NPs, and MgO NPs [34], and hypericin [35,36].

The HY is a naturally occurring photosensitizer that has proven to be non-toxic to cells but favoring healing of experimental CL in hamsters infected by *L. (V) panamensis* [37]. However, HY can easily form heavyweight molecular aggregates in aqueous systems, easily losing its activity[38]. Beside, HY is a lipophilic compound which requires organic solvent or ethanoic solutions restricting its medical applications [39]. Attempts have been made to decrease the formation of aggregates and increase the efficiency of HY uptake with encapsulation in polymeric or liposomal systems [40], representing high economic value. For this reason, calcium phosphates are identified as possible encapsulant products. Calcium phosfates nanocarries emerge as an interesting alternative to improve the selectivity and release of the photosensitizing in the desired cells. Beside, nanoparticles help drugs overcome the physiological and biological barriers and improve cell uptake.

The application of CP as encapsulating systems depends mainly on their microstructural and morphological properties, which in turn are related to the mechanisms of production. Different synthesis routes for obtaining calcium phosphates have been explored. They include solid-state, hydrothermal, co-precipitation, sol-gel, and combustion in solution routes. [41–44]. The combustion in solution is a route with easy implementation that allows obtaining diverse structures, including those with nanometric characteristics. This route is based on a redox reaction between a fuel and an oxidizer, releases large amount of heat when it reaches the appropriate thermodynamic conditions. The phenomenon occurs in presence or not of flame and produces large amounts of NO_x and CO_2 obtaining the final stable or metastable phases without the need for subsequent heat treatment [45]. The obtained nanometric and porous structures are capable of encapsulating compounds by adsorption and/or absorption.

In this work we studied the potential use of calcium phosphates as HY encapsulant, a liphophilic and photodynamic compound, in the CL treatment.

2. Experimental procedures

2.1. Synthesis and characterization of calcium phosphates

The CP nanostructures were synthesized via self-combustion. Calcium nitrate tetrahydrate $Ca(NO_3)24H_2O$ (Merck), and di-ammonium hydrogen phosphate $(NH_4)_2HPO_4$ (Alfa Aesar) were used as raw material sources of calcium and phosphorus, respectively. Glycine $C_2H_5NO_2$ (Merck) was used as fuel and 65% nitric acid HNO_3 (Biochemical) as a catalyzer. Initially, $Ca(NO_3)_24H_2O$ and $(NH_4)_2HPO_4$ were mixed in 20 mL of distilled water under constant stirring, maintaining a ratio of Ca/P = 1.5. Sufficient HNO_3 was added to the mixture to dissolve the previously-formed precipitate. In order to add the appropriate amount of fuel (glycine) to obtain the maximum combustion temperature, the parameter φ was used, defined as $\varphi = \frac{\sum \text{Coefficient . Reducingel ements X Valence}}{\sum \text{Coefficient . Oxidantel ements X Valence}}$ and adjusted to the stoichiometric ratio oxidative-fuel for φ = 1[28]. At this stage, the solution was stirred for one hour and heated to a temperature of 60 °C up to near-total evaporation of the solvent, in order to form a resin. Subsequently, the temperature was increased to 140 °C until combustion. The ash formed was heat-treated at 800 °C for 2 h. The powders were suspended in 80 mL of isopropyl alcohol and ultrasonicated on a 500W Cole Palmer kit at different amplitudes for 1 h. The powders were again dried for 2 h at 90 °C. The obtained powders were evaluated using X-ray diffraction with a double circle multipurpose Xpert-Pro PANanalytical Radiation Cu-Ka (λ = 0.15406 nm) diffractometer in a range of diffraction of 10–50° (2θ). Raman spectroscopy measurements were obtained using Labram Horiba equipment with a laser of 532 nm and a power of 50 mW. The morphology of the powder was analyzed by means of field-emission microscopy and scanning electron microscopy in JEOL and Carl Zeis equipment, respectively.

2.2. Hypericin loading in the calcium phosphate nanostructures and quantification of the amount of charged hypericin

For the encapsulation of HY, the impregnation-agitation technique was used. 20 mg of CP nanoparticles were re-suspended in 0.5 mL of dimethyl sulfoxide (DMSO) and ultrasonicated for 30 s. A solution of HY in DMSO with a concentration of 100 μM was separately prepared and subsequently added to the nanoparticle dispersion. The system was stirred for 20 h at 60 rpm. After this, the sample was subjected to centrifugation at 15000 r.p.m. and the precipitate was recovered. The possible interactions of the HY with the encapsulant CP nanoparticles were evaluated by IR spectroscopy with Fourier transform (Shimadzu IRTracer-100) and UV/VIS spectrophotometry (ITecan M410).

To determine the amount of HY loaded in the CP nanoparticles, a standard curve of concentration was obtained. The HY was diluted in methanol in serial dilutions 1:5 starting at 25 μg of HY. The fluorescence of the dilution was read on a Varioskan Flash (Thermo Scientific) spectrofluorometer at an excitation wavelength of 542 nm and emission at 610 nm. The experiment was performed in triplicate. The obtained data were adjusted to the best mathematical model, obtaining an equation of intensity according to the concentration. Subsequently, 20 μg/mL of HY-loaded particles were washed in methanol, vortexed, and centrifuged several times, in order to release the HY contained therein. The supernatant was recovered and its fluorescence measured three times. These results were compared to the standard curve previously determined to calculate the amount of HY encapsulated in the CP nanoparticles.

2.3. Culture of human macrophages derived from peripheral blood monocytes

For the *in vitro* biological experiments, the human macrophages derived from peripheral blood monocytes (huMDM) were obtained, the methodology previously reported [37]. For this, 60 mL of periferal blood were taken using syringe and needle #21G. The blood was transferred to a polypropylene conical tube containing glass pearls (2 pearls per mL of blood) and carefully shaken manually until fibrin clot was formed. After the defibrination process was completed, the defrinated blood was transferred to a new tube, where it was diluted with PBS in a 1: 1 ratio. Three mL of Ficoll-Hystopaque 1077 (Sigma-Aldrich) was placed in 15 mL conical tubes, and 12 mL of the diluted blood was slowly added to the Ficoll. These tubes were centrifuged at 2000 r.p.m. (300 g) for 20 min at room temperature. The mononuclear cell layer was extracted and washed twice with PBS at 1800 r.p.m.

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