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Hybrid nanofibers based on poly-caprolactone/gelatin/hydroxyapatite nanoparticles-loaded Doxycycline: Effective anti-tumoral and antibacterial activity

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ABSTRACT

Cancer is one of the leading causes of morbidity and mortality Worldwide, 19.3 million new cancer cases are expected to be identified in 2025. Among the therapeutic arsenal to cancer control one could find the Doxycycline and the nano hydroxyapatite. The Doxycycline (Dox) not only shown antibiotic effect but also exhibits a wide range of pleiotropic therapeutic properties as the control of the invasive and metastatic cancer cells characteristics. The purpose of the present study was to evaluate both cytotoxicity *in vitro* and antibacterial activity of electrospun Dox-loaded hybrid nanofibrous scaffolds composed by hydroxyapatite nanoparticles (nHA), poly-ε-caprolactone (PCL) and gelatin (Gel) polymers. Both nHA and Dox were dispersed into different PCL/Gel ratios (70:30, 60:40, 50:50 wt%) solutions to form electrospun nanofibers. The nHA and Dox/nHA/PCL-Gel hybrid nanofibers were characterized by TEM microscopy. *In vitro* Dox release behavior from all of these Dox-loaded nHA/PCL-Gel nanofibers showed the same burst release profile due to the high solubility of Gel in the release medium. Antibacterial properties of nanofiber composites were evaluated using Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Porphyromonas gingivalis* (*P. gingivalis*) bacteria. The co-delivery of nHA particles and Dox simultaneously exhibited inhibition of bacterial growth more efficiently than the delivery of either Dox or nHA at the same concentrations, indicating a synergistic effect. The results showed that cancer cell tested had different sensibility to co-delivery system. On the whole, A-431 cells were found exhibited the most pronounced synergistic effect compared to CACO-2 and 4T1 cancer cells. Based on the anticancer as well as the antimicrobial results in this study, the developed Dox/nHA/PCL-Gel composite nanofibers are suitable as a drug delivery system with potential applications in the biomedical fields.

1. Introduction

Cancer is still one of the leading causes of morbidity and mortality worldwide mainly due to drawbacks in treatment-associated, recurrence rates, metastasis and late diagnosis. The metastasis is a major factor to the deaths of cancer patients thus the one of the main focus of the new therapies is to prevention or to control the metastatic process [1]. In this context, combination therapies with different mechanisms of drug action become an attractive route against chemotherapeutic resistance and proliferation cycles.

The Doxycycline hyclate (Dox) is a well-known antibiotic with broad-spectrum belongs to the tetracycline family. Bacteriostatic effects of tetracyclines are associated to inhibition of protein synthesis, by preventing the binding of the aminoacyl t-RNA to 30S ribosomal subunit [2–4]. Moreover, studies have reported that Dox can exhibit other biological actions such as antiproliferative, antiangiogenic, anti-collagenolytic, anti-inflammatory, osteoclast-inhibitory, and fibroblast-stimulatory properties [5]. This antiproliferative activity of the Dox is attributed to the inhibition of matrix-metalloproteinases (MMPs), enzymes involved in the degradation and remodelation of the extracellular matrix [6,7]. Herein, the Dox ability in the MMPs inhibition can represent a new potential targets for treatment and detection of cancer patients [8,9]. In addition, anti-cancer of Dox against different carcinoma cells as melanoma, lung, breast, colon and prostate cancers have

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been confirmed by *in vivo* studies increasing the potential use of this drug in cancer therapies [5,10,11].

On the other hand, the hydroxyapatite (HA, $(Ca_{10}(PO_4)_6(OH)_2)$ is the most widely accepted inorganic nanomaterial for use in clinical due to its excellent biocompatibility property and adequate biodegradation. The HA nanoparticles (nHA) has found success as delivery vehicles for antibiotics, nucleic acids, proteins and anticancer drugs due to its high surface area to volume ratio, high surface activity and strong ability to absorb a variety of chemical species. In addition, nHA themselves have been reported to inhibit the proliferation of several kinds of cancer cells, such as osteosarcoma, hepatoma, gastric cancer, colon cancer, melanoma, breast cancer, and glioma cells while supporting proliferation to the normal bone cells [12]. These studies have shown that the size and morphology of the HA can determine the biological behavior and induce differential cytotoxicity on cancer cells [12,13]. Thus, the n-HA/Dox combination therapy can represent new combined agents with potential antitumoral effect and different mechanisms of action for the cancer combat.

In addition, the localized therapy, either for curative or palliative intent, could supplement or replace existing treatments as local drug delivery to control the tumor growing or metastatic process [14]. Thus, fabricate scaffolds with desired properties for specific tissue regeneration and drug delivery have been evaluated to encapsulate drugs into the electrospun nanofibers from natural and synthetic polymers [15] offering a local treatment of the tumor [10,16,17]. The electrospinning is one of the most used and efficacious technique to generate electrospun fibers from materials of diverse origins with micro-to nano-meter range diameters by using high-voltage electrostatic fields [18]. Blending natural and synthetic polymers offer the advantage to fine-tune the desired properties of the electrospun scaffolds [19]. Thus, the presence of natural polymer enhances cell attachment, biocompatibility and bioactivity to the scaffolds. In the field of drug delivery, polymeric blends nanofibers provide many opportunities to design and adjust release kinetics of a large number of antitumor and antibacterial agents [20,21]. For instance, the poly-ε-caprolactone (PCL) is a hydrophobic, bioresorbable and biocompatible synthetic polyester approved by the Food and Drug Administration (FDA) in biomedical devices for applications in drug delivery and tissue repair [22,23]. On the other hand, gelatin (Gel) is a natural biopolymer derived from the partial hydrolysis of collagens that has low cost, natural abundance, great biodegradability and biocompatibility in physiological environments as well as a well-established employment in the food and pharmaceutical industry [24].

The literature has reported the electrospun PCL/Gel composite scaffolds as a versatile synthetic-natural polymer blending for tissue-engineering applications such as wound healing and cartilage, muscle, neural and blood vessel repair [24–29]. In order to develop useful tailored biomaterials, electrospun nanofibers are often functionalized, entrapping a variety of bioactive substances within the interior or physically immobilized on the surface for controlled drug delivery and enhance cell adhesion [30–34]. In this context, a polymer combined with inorganic nanoparticle as the hydroxyapatite (HA) have been engineered to be used for bone regeneration, but all afore mentioned knowledge has never been involved as an efficient anti-cancer system and drug delivery.

In the present study, we aimed the Dox and nHA release from the biodegradable polymer composite nanofibers of PCL/Gel that could be used as a strategic local drug delivery. Dox and nHA were encapsulated into various PCL/Gel ratios (70:30, 60:40, 50:50 wt%) solutions and the Dox/nHA-loaded PCL-Gel composite nanofibers were prepared by electrospinning. The nHA and Dox/nHA/PCL-Gel composite nanofibers were characterized using different techniques. The antimicrobial activity of the Dox/nHA/PCL-Gel nanofibers was investigated using *Staphylococcus aureus* (*S. aureus*) and *Porphyromonas gingivalis* (*P. gingivalis*) as a model bacterium on solid medium. The antitumor activity of the Dox/nHA/PCL-Gel nanofibers was determined through *in vitro* using three different tumor cell lines: murine mammary carcinoma 4T1, epidermoid carcinoma A-431 and human colon adenocarcinoma CACO-2, exposed to the release medium of composite nanofibers with different Dox and nHA concentrations.

2. Experimental

2.1. Materials

PCL (Mw = 43,000–50,000 g/mol) and Doxycycline hyclate were purchased from Polysciences, Inc. (USA) and Drogaria Araujo S.A. (Brazil), respectively. Gelatin (from bovine skin type B), glacial acetic acid (99.9%), ethyl acetate (anhydrous, 99.8%), ammonia (25–30%), calcium nitrate and sodium phosphate dibasic anhydrous were obtained from Vetec/Sigma-Aldrich (Brazil). All the others materials used were analytical grade. *Staphylococcus aureus* (*S. aureus*) and *Porphyromonas gingivalis* (*P. gingivalis*) were obtained from American Type Culture Collection (ATCC). Mouse embryo fibroblast (3T3-L1), murine mammary carcinoma (4T1), epidermoid carcinoma (A-431), human colon adenocarcinoma (CACO-2) cell lines were kindly provided by Rio de Janeiro Cell Bank (RJCB) (Brazil). Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, fetal bovine serum (FBS), antibiotic and antimycotic solution (10.000 units/mL of penicillin, 10.000 μg/mL of streptomycin) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibco (USA). Phosphate buffer saline (PBS) pH 7.4 was prepared freshly.

2.2. Preparation of hydroxyapatite nanoparticles

The synthesis of nHA was following a co-precipitation reaction as previously method described in the literature with minor modifications [35]. In brief, 9.45 g calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$, 0.04 mol) was dissolved in 100 mL of distilled water and stirred vigorously. In 60 mL of distilled water, 3.41 g sodium phosphate dibasic (Na_2HPO_4, 0.024 mol) were dissolved and the pH of the solution was adjusted to 10 with ammonium hydroxyde (NH_4OH). The Ca^{2+} solution obtained was then added drop-wise into phosphate solution. Then the mixture was continuously stirred and refluxed in a silicon oil bath at 90 °C for 24 h. The white precipitate produced was filtered with distilled water and subsequently dried in an oven at 100 °C for 24 h.

2.3. The preparation of electrospinning solutions

The PCL-Gel (PG) blends, nHA/PCL-Gel (PGHA) composites and drug-loaded Dox/nHA/PCL-Gel (PGHAD) composites with different PCL/Gel ratios were obtained by electrospinning. PCL and Gel were dissolved in a co-solvent mixture of acetic acid, ethyl acetate, and water (3:2:1 wt) to prepare polymer solutions with concentration of 16 wt% with following weight ratio for PCL/Gel 70:30 (PG1), 60:40 (PG2) and 50:50 (PG3). The solutions were stirred for 10 h at 45–50 °C and then immediately electrospun, as described by Binulal [36]. For the generation of the PGHA composite systems, different ratios of PCL/Gel solution in co-solvent were mixed with a previously sonicated dispersion of nHA (9 wt% relative to) and after electrospun the nanofibrous composites were denoted to PGHA1 (70:30), PGHA2 (60:40) and PGHA3 (50:50). All the drug-loaded Dox/nHA/PCL-Gel (PGHAD) composites were obtained with 2.8 wt% of Dox labeled as PGHAD1 (70:30), PGHAD2 (60:40) and PGHAD3 (50:50).

2.4. Electrospinning process

The experimental setup used for electrospinning process of this study was optimized by changing different parameters. The best electrospinning conditions were set as follows: each polymer solution was loaded in a 5 mL plastic syringe fitted with a stainless steel needle with an inner diameter of 0.4 mm. The distance between the tip of the needle