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PLA/chitosan/keratin composites for biomedical applications

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ABSTRACT

Novel composites based on PLA, chitosan and keratin was obtained via blend preparation. The goal of this contribution was to evaluate mechanical and in vitro behavior of the composites. The results point out composites with improved Young modulus and decreased tensile strength, significant increase in hardness (compared to PLA) and a good uptake of the surface properties. Biological assessments using human osteosarcoma cell line on these composites indicate a good viability/proliferation outcome. Hence preliminary results regarding mechanical behavior and in vitro osteoblast response suggest that these composites might have prospective application in medical field.

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1. Introduction

The interest for "green" materials with medical applications was increased due to both patients and medical world that search for solutions to their challenges such as the need for substitutes to replace or repair tissues or organs problems.

The most biodegradable materials comprise synthetic polyesters such as poly(L-lactic acid) and poly(L-glycolic acid) and natural polymers such as chitosan, alginate, collagen, and fibrin [1]. Also, some inorganic materials e.g. hydroxyapatite or certain glasses have been used to obtain materials for hard tissue applications [2]. The interest for polyesters is due to their hydrolysable ester bonds and one of the most important constituent of this class is polylactic acid (PLA) which is derived fully from renewable resources. It has been used in biomedical applications but its application is somewhat limited by its inherently poor properties such as reduced impact strength and low thermal stability [3].

Poly(L-lactic acid) has been widely studied for use in biomedical applications such as sutures, scaffolds for tissue engineering, orthopedic devices, or drug delivery systems due to its biocompatibility and bioresorbability [4,5].

Chitosan is a polysaccharide obtained by the deacetylation of chitin and has many applications due to its price, excellent oxygen barrier properties, antimicrobial effects, biodegradability, biocompatibility, antimicrobial activity and non-toxicity [6,7]. Due to easy processing method one may obtain films, fibers, gels and foams, as well as beads of different sizes and morphology with medical applications [8]. One important property is the fact that chitosan interacts with cells and cellular lysozyme degrades chitosan in vivo [9]. In the same time, various kinds of chitosan derivatives have medicine applications e.g. bone, cartilage, skin, nerve and blood vessel [7,10].

Literature data reported a good biocompatibility of PLA [9,11]. That is why, it is used in biomedical applications as internal body components, for implants and drug delivery systems [12]. Keratin is the major component of feathers. It is a structural protein characterized by high cystine content and a significant amount of hydroxyl amino acids, especially serine [13]. It's characterized by the presence of a range of noncovalent interactions (electrostatic forces, hydrogen bonds, hydrophobic forces) and covalent interactions (disulphide bonds), which are difficult to be damaged.

The recent trends in biodegradable polymers indicate new development strategies and engineering to achieve polymeric materials with great interest both in the academic and industrial fields. It was found that the incorporation of functional fillers in the PLA matrix could improve the physical properties, as well as the surface characteristics of the matrix that are important for tissue engineering and artificial bone reconstruction. Motivated by our preliminary results [14], the purpose of this work is to investigate PLA–chitosan–keratin composites as biomaterials with potential applications in medicine, by means of mechanical and in vitro studies. Preliminary results regarding mechanical behavior and in vitro osteoblast response confirmed their potential for medical applications.

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2. Materials and methods

2.1. Preparation of the composite film

Before blend preparation, PLA (type 2002D, supplied by NatureWorks) pallets, chitosan (produced by Vanson Inc. with an average molecular weight of 1200 kDa and acetylation degree of 34%) and feather fibers were dried in a vacuum oven for 6 h at 50 °C. Compounding components were performed at 175 °C for 10 min at a rotor speed of 60 rpm using a fully automated laboratory Brabender station. Literature data [15] show that the addition of chitosan did not influence thermal properties and degree of crystallinity of PLA. Specimens for the mechanical characterization were prepared by compression molding using a Carver press. The compression molding was carried out at 175 °C with a pre-pressing step of 3 min at 50 atm and a pressing step of 2 min at 150 atm. A neat PLA sheet was prepared in the same conditions and acted as a reference. Composition and preparation of the samples are as follows: A111: 70% PLA and 30% chitosan; A121: 68% PLA, 30% chitosan and 2% keratin; A131: 66% PLA, 30% chitosan and 4% keratin.

2.2. DSC analysis

Thermal characterization of composites has been performed with a TA Instruments Q20 Dynamic Scanning Calorimeter. All the samples were heated from 25 °C up to 200 °C with 10 °C/min, kept for 2 min and then cooled down to 25 °C with a cooling rate of 5 °C/min. An empty crucible was used as a reference material. All measurements were performed under N2 atmosphere. The degree of crystallinity of the PLA samples was obtained by dividing the melting enthalpy of the sample by 93.7 J/g [16], which is the estimated melting enthalpy of a pure PLA. The crystallinity of the composite materials was estimated as the function of PLA fraction in the composite and the melting enthalpy.

2.3. Characterization of composites

Mechanical tests in terms of tensile and impact strength were performed.

Thus, tensile strength measurements were carried out following ISO 527-2000 standard method, using an Instron 5 kN test machine operated at a crosshead speed of 30 mm/min. The unnotched Charpy impact strength was measured according to ISO 179-2010 using a Ceast apparatus provided with a hammer of 15 J. Seven specimens were tested for each material.

The Vickers hardness tests were performed with a Shimadzu microhardness tester. A constant load of 4.903 N was applied for 12 s for all composite samples. Ten tests have been carried out for each sample and the average value is given.

2.4. Biological tests

2.4.1. Cell culture

MG63 osteoblast-like cells (ATCC® no CRL-1427™, Rockville, MD-USA) were cultured in Dulbecco's modified Eagle's medium with 4500 mg L^{-1} glucose (DMEM) from Gibco supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax I (Life Technologies) and 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin. Cell cultures were sustained at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air. The samples were sterilized using gamma irradiation according with ISO 11137—2.0 kGy at room temperature [17] and before using them in cell culture studies the samples were submerge in a diluted ethanol solution (70% v/v) for 15 min, followed by intensive washing in sterile phosphate buffer saline (PBS) solution. The cells were seeded over the sterile samples at a density of 3.0×10^4 cells per cm^2 in a 48-well plate (Cellstar®, Greiner Bio-One) up to 7 days. Standard 48-well tissue culture plates (polystyrene) were used as control surface. Cells were maintained under standard cell culture conditions (5% CO_2, 95% humidity and 37 °C). The medium was changed every 2–3 days. Control cultures and seeded material samples were evaluated at days 1, 2 and 3 for cell viability/proliferation and at days 3 and 7 the samples were investigated via confocal laser scanning microscopy (CLSM; Leica SP2).

2.4.2. Cytotoxicity assay

CellTiter 96®AQueous One Solution Cell Proliferation (Promega, Madison, WI) assay was used to study cell viability. The metabolic cell activity (an indirect measure of cytotoxicity) was measured by the conversion of MTS to formazan, which can be photometrically detected. MTS was mixed with fresh medium at the ratio of 1:10 and added to the cells for 1.5 h. The cells were placed in a CO_2 incubator at 37 °C. After incubation time supernatants were transferred to a new microplate and optical density was measured photometrically at 492 nm in an ELISA 96 well-plate reader. All experiments were performed in triplicate and were treated and represented by their mean value and standard deviation parameters.

The percentage cell viability was calculated according to the following equation:

$$\%\ \text{cell viability} = 100 \times \left(\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}\right),$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of cells tested with various formulations and $\text{Abs}_{\text{control}}$ is the absorbance of reference cells (incubated on the culture media only).

2.4.3. Immunofluorescence analysis

The cells were seeded 3.0×10^4 cells per cm^2 in a 48-well plate. After preset time intervals, immunocytochemical staining was performed on whole samples. Briefly, the samples with cells were washed with PBS and fixed using a solution of 3.7% v/v paraformaldehyde. The fixed cells were permeabilized with buffered 0.5% v/v Triton X-100. Subsequently the cells were stained for nuclei with DAPI (0.4 μg mL^{-1}) and cytoskeletal organization was revealed by actin labeling. F-actin filaments were stained with tetramethylrhodamine isothiocyanate (TRITC) conjugated phalloidin 0.2 μg mL^{-1} (Sigma, St. Louis, MO, USA). Labeled samples were examined by CLSM.

2.5. Statistics

Statistical calculations and analyses were performed with the use of Prism 5 (GraphPad Software, Inc.) statistical software package. One-way analysis of variance (ANOVA) was employed to assess the statistical significance of results at a probability of error of 5% (*), 1% (**) and 0.1% (***). All experiments were repeated at least three times, and the results are presented as mean ± standard deviation (SD).

3. Results and discussions

3.1. DSC results

The thermal behavior of the studied materials is presented in Fig. 1 and Table 1.

Pure chitosan does not have melting properties and hence no endothermic peaks associated to melting process were detected, as other authors reported [18]. The thermogram of neat PLA revealed a glass transition temperature at 59.4 °C followed by a cold crystallization process peak at 127.8 °C and then melting peak at 152.97 °C, with an enthalpy of fusion of 13.62 J/mol. It was found that the addition of chitosan determined an increase of Tg to 60.2 °C, which can be explained by chitosan hindering movements of PLA chains.

The presence of chitosan, which is a semicrystalline polymer, determines a loss of crystallinity in the PLA matrix, which drops from 20.61%

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