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Functionalization of Biodegradable Polyester for Tissue Engineering Applications

Rossana M. S. M. Thiré,[*1] *Taíla O. Meiga,*[1,2] *Sabrina Dick,*[2] *Leonardo R. Andrade*[2]

Summary: Chemical modification of polymer surface may potentially be used to create smart materials that can guide cellular adhesion, proliferation and maintenance of specific expression of molecules. The microbial polyester poly (3-hydroxybutyrate) (PHB) has been attracted attention as promising material for applications in tissue engineering. In this work, a wet-chemical method, base ethylenediamine aminolysis, was performed to improve the adhesion of chondrocytes isolated from human articular cartilage to PHB films. The effects of chemical treatment on PHB films was evaluated by following changes in morphology and surface chemical composition using atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS), respectively. While the effect on cells morphology was studied by scanning electron microscopy (SEM). The treatment with ethylenediamine did not change significantly the morphology of the structures of PHB films surface. However, the roughness of the aminolyzed films was slightly higher. The introduction of nitrogen-containing groups was confirmed by XPS. *In vitro* experiments indicated that the surface modification did not have toxic effects in cells, since they could adhere and proliferate on modified PHB films. It was observed that long-time treatment improved ability of PHB films to support cell growth, which could be accounted to physicochemical and topological effects.

Keywords: biomaterials; poly(3-hydroxybutyrate); surface modification; tissue engineering

Introduction

The design and development of novel materials for tissue engineering have attracted much interest in recent years. Tissue engineering aims to heal the damaged tissue using a compatible biomaterial alone or cellularized. Tissue engineered scaffolds are generally three-dimensional biodegradable structures that support temporarily cell adhesion, proliferation, differentiation and synthesis of specific extracellular matrix (ECM); allowing the cells to regenerate the tissue.[1] An approach of tissue engineering science includes the application of biodegradable polymers-based scaffolds.

Polyhydroxyalkanoates (PHA) are polyesters produced by microorganisms under unbalanced growth conditions. They are generally biodegradable and thermoprocessable, making them attractive as biomaterials for applications in both conventional medical devices and tissue engineering.[2] Poly(3-hydroxybutyrate) (PHB) is one of the most widely investigated member of PHAs family. PHB is partially crystalline and possesses some mechanical properties close to those of isotactic polypropylene.[3] *In vitro* tests have shown that PHB is biocompatible to various cell lines, including osteoblasts and rabbits chondrocytes.[4] Therefore, this polymer has potential therapeutic applications such as matrix for *in vitro* proliferous cells (scaffolds), sutures,

[1] PEMM/COPPE, Universidade Federal do Rio de Janeiro, PO Box 68505, 21945-970, Rio de Janeiro, RJ, Brazil
E-mail: rossana@metalmat.ufrj.br
[2] Departamento de Histologia e Embriologia, Instituto de Ciências Biomédicas, CCS, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, RJ, Brazil

implantable rods for the local delivery of drugs, etc.[2,3]

Surface properties of any medical device are extremely important, since they affect the biological interactions and responses across the interface with host tissue. Biomaterial surface properties influence protein adsorption and elicit diverse cellular responses in biomedical and biotechnology applications *in vivo* and *in vitro* experiments. Hydrophobicity plays a major role in the attachment of proteins to biomaterials. However, other interactions like electrostatic charges, dipole interactions, hydrogen bonding, as well as sterical and entropical forces have also to be considered as providing important contributions.[5] Thus, it is not only necessary for the biomaterial to be biocompatible and biodegradable, in tissue engineering applications, it is also essential that the surface be conducive to cell attachment and subsequent tissue growth and regeneration. Tailoring surface properties of degradable polymers is key to the progress of various tissue engineering strategies.[6] This is often accomplished by immobilizing specific proteins or peptide sequences on the scaffold surface that mimic natural components. The materials improved with bioactive molecules can be used as smart scaffold that potentially acts as an artificial ECM to guide cell adhesion and consequently tissue formation.

Surface modifications fall into two main categories: (1) chemically or physically altering groups, compounds or molecules of an existing surface (chemical modification, etching, mechanically roughening) or (2) overcoating existing surface with a material having a different composition (coating, grafting, thin film deposition).[7] Chemical modifications of polymeric surfaces include direct chemical reaction with a solution (chemical wet- treatment), which can involves chemical oxidation, hydrolysis and aminolysis. These treatments can modify the surface reactivity by altering surface charge, chemical group functionality and wettability.[8]

In order to trigger the cell-matrix adhesion on PHA surfaces several modification techniques have been recently applied including alkaline hydrolysis, lipase treatment and plasma treatment.[9–12] Some of these treatments were followed by protein immobilization onto the polymeric surface.

In this work, a wet-chemical method, based on ethylenediamine aminolysis, was evaluated in order to introduce hydroxylic and amine groups onto the surface of PHB films. It is expected that the introduction of this functional groups would improve chondrocytes adhesion and proliferation. Besides, the creation of active groups that allow covalent attachment of adhesive proteins or cell adhesive peptides is a pre-requisite to the achievement of bioactive surfaces, which would stimulate a suitable cellular response in the presence of biomaterial.

Experimental Part

Poly(3-hydroxybutyrate) (PHB), in the form of a white powder, with an weight average molecular weight (M_w) of 524,000 Da and polydispersity index of 8.6, was supplied by PHB Industrial S/A (São Paulo, Brazil). Films were prepared by dissolving PHB in chloroform for approximately 2 h at 65 °C. The resulting polymer solution (10% w/v) was vacuum filtered using medium speed filter papers. Five milliliters of the solution was spread on a circular glass mold and allowed to dry at room temperature overnight. PHB films were peeled off and conditioned in a desiccator containing silica gel at room temperature and atmospheric pressure for approximately 4 days (until weight constance) to allow the complete evaporation of the solvent. PHB discs were 0.2 mm in thickness and 1.4 mm in diameter, approximately (digital micrometer Mitutoyo n° 293–265, Mitutoyo Corp.).

For aminolysis treatment, the obtained PHB films were submerged into 0.1 M ethylenediamine solution at 50 °C for 45–120 min. After treatment, the films were exhausted rinsed with deionized water at room temperature to remove the excess of ethylenediamine and dried in a desiccator containing silica gel at room temperature and atmospheric pressure.

Scanning probe microscopy analysis was carried out with a Topometrix Acurrex-IIL system. Morphology of the untreated and treated PHB films was analyzed in intermittent contact mode using silicon tips (MikroMasch™ NSC16) mounted on a cantilever with a spring constant of ca. 40 N/m and resonance frequencies in the range of 170 kHz. Samples were fixed on double-coated adhesive tapes and the atomic force microscopy (AFM) images were obtained in air. Surface chemical elemental composition was evaluated by X-ray photoelectron spectroscopy (XPS). This analysis was performed using a Phoibos 100 spectrometer equipped with a monochromatic MgKα source. Elements present were identified from survey spectra recorded at 50 eV pass energy. High-resolution spectra were recorded from individual peaks at 20 eV pass energy.

In vitro tests were performed using chondrocytes isolated from human articular cartilage to evaluate the biocompatibility of the films. The morphology of the cells which were adhered to untreated and treated films surface was evaluated by scanning electron microscopy (SEM). Approximately 4×10^5 cells were seeded on untreated and treated PHB films (sterilized in ethylene oxide) in 24-well plates in Dulbeco's Modified Eagle Medium (DMEM, Gibco), and maintained for 7 days at 37 °C in a humid atmosphere (95% Relative Humidity) containing 5% CO_2. The culture medium was changed every 2 days. After this period, the adhered cells were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 hour, rinsed in the same buffer and dehydrated in graded ethanol series. The samples were dried by liquid CO_2 critical-point method (Baltec CPD 020), deposited on conductive carbon tape covered aluminum stubs, and gold-sputtered. The samples were observed under a Jeol JSM 6460LV electron microscopy at an acceleration voltage of 10 kV.

Results and Discussion

The surface of poly(3-hydroxybutyrate) (PHB) films was modified by aminolysis with 0.1 M ethylenediamine for 45–120 min in order to improve the chondrocytes adhesion to these materials. Aminolysis is a wet-chemical treatment which involves the reaction of one amino group from diamine with the ester group from PHB to form a covalent bond, -CONH- and/or $-CONH_2$, while the other amino group is unreacted and free. Hydroxyl-terminated chains will also be yielded on the polyester surface during this process.[13]

Figure 1 shows the X-ray photoelectron (XPS) spectra of the PHB films surfaces prior (a) and after aminolysis for 120 min (b). As expected, XPS spectra reveals that small amount of nitrogen-containing groups reacted with the surface after long-term treatments. These PHB surfaces aminolyzed were found to contain approximately 2 at.% nitrogen. No significant change in oxygen:carbon ratio was detected for treated films. It was observed that silicon was present at the surface of untreated film. This element was not detected in XPS spectra for PHB treated films. This fact may indicate that besides functionalization also an erosion of the surface can occur during 120 min of aminolysis treatment.

The effect of chemical modification on the morphology of PHB films was evaluated by atomic force microscopy (AFM). Three-dimensional AFM topographic images of untreated and treated PHB films are shown in Figure 2. The surface of untreated PHB films consists of oval-shaped structures with 2 μm in major axis, in average (Figure 2a). The treatment with ethylenediamine did not change significantly the dimensions of the structures in treated films (Figure 2b–d). However, the vertical scales of the AFM images show that the maximum height of structures increased from 579.91 nm (Figure 2a) to 665.7 nm (Figure 2b) after aminolysis for 45 min. When the maximum height of structures of aminolyzed films was compared (Figure 2b–d), no significant difference was detected.

Intermittent contact mode AFM allows the use of the changes in phase angle of the cantilever probe to produce phase contrast images. These images provide significantly

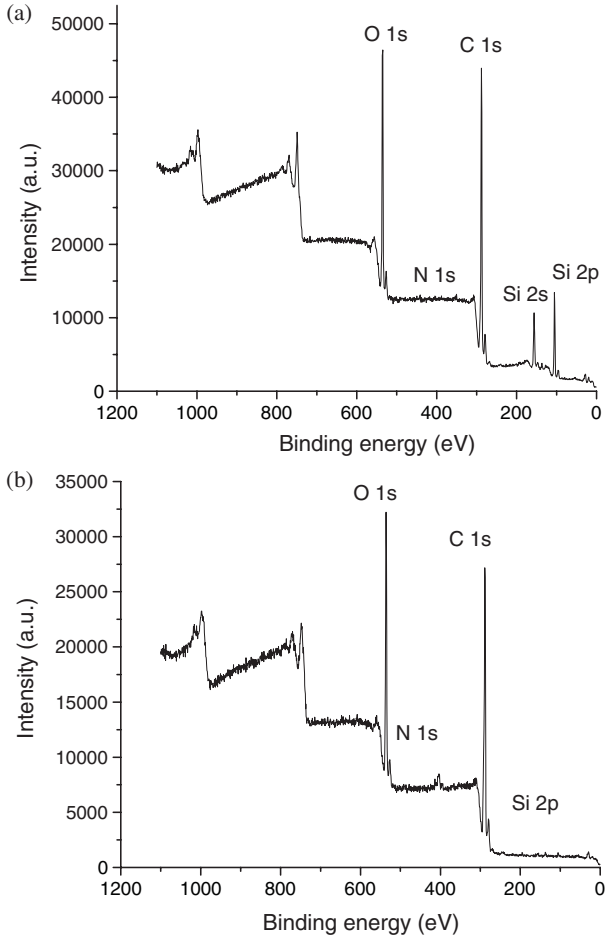

Figure 1.
The XPS spectra of untreated PHB films (a) and PHB aminolyzed with 0.1 M ethylenediamine for 120 min.

more contrast than topographic images and have been shown to be sensitive to material surface properties, such as stiffness, viscoelasticity and chemical composition.[14] AFM phase contrast image at higher magnification (Figure 3) showed that some structures of the PHB films (indicated by arrows) did not present a smooth surface after 120 min of treatment. Probably, this surface modification may be related to the erosion process caused by ethylenediamine treatment, as suggested by XPS results.

AFM also allows accurate measurements of the roughness of materials surface. The root mean square roughness (RMS roughness) of untreated and treated PHB films was calculated from 5 $\mu m \times 5$ μm AFM topographic images. The results are presented in Table 1. The roughness of the treated films was slightly higher. However, it was not observed a direct relation between the increasing in roughness and the treatment time.

Surface modifications should contribute to adsorption of proteins that participate in cellular adhesion to biomaterial. The cellular adhesion to substrate is an important parameter and a pre-requisite in understanding the biocompatibility of newly developed biomaterials. Human chondrocytes were cultured in direct contact with the untreated and aminolyzed films and the

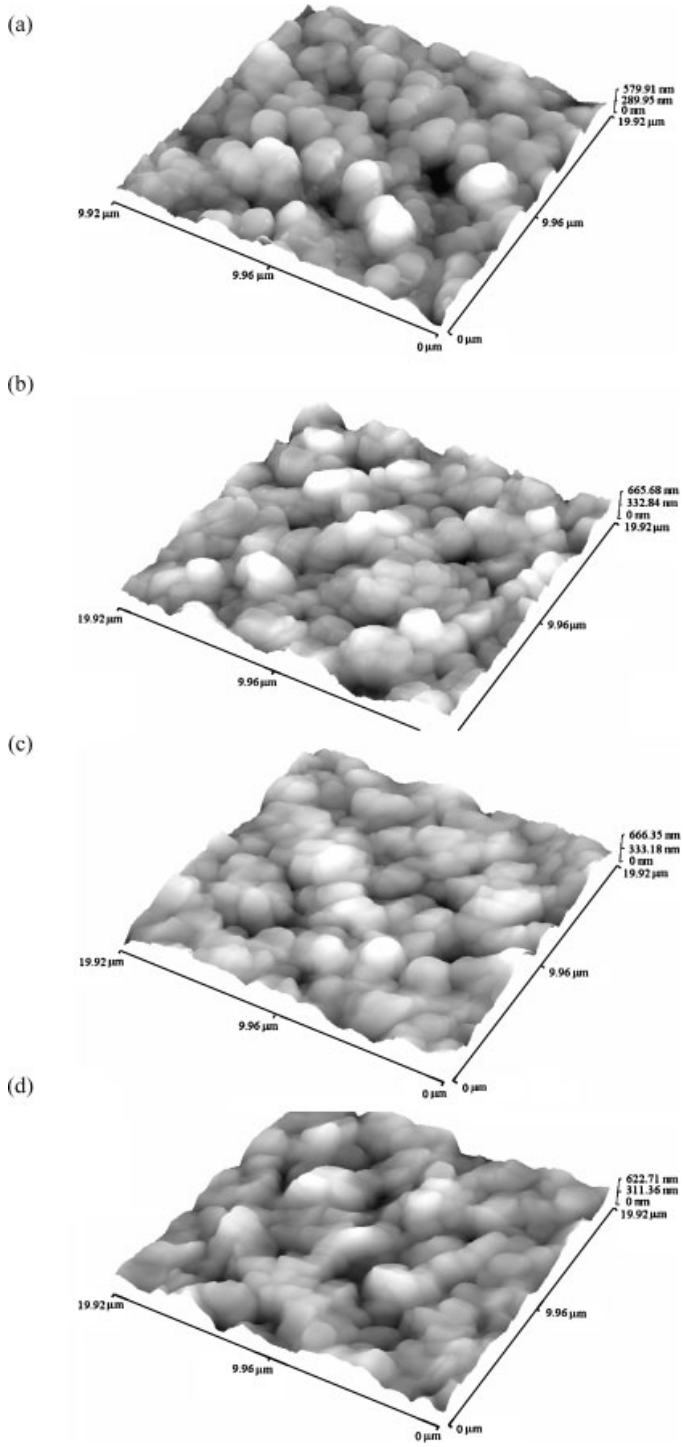

Figure 2.
AFM three-dimensional topographic images of untreated (a) and aminolyzed PHB films with 0.1 M ethylenediamine for (b) 45 min, (b) 90 min and (c) 120 min.

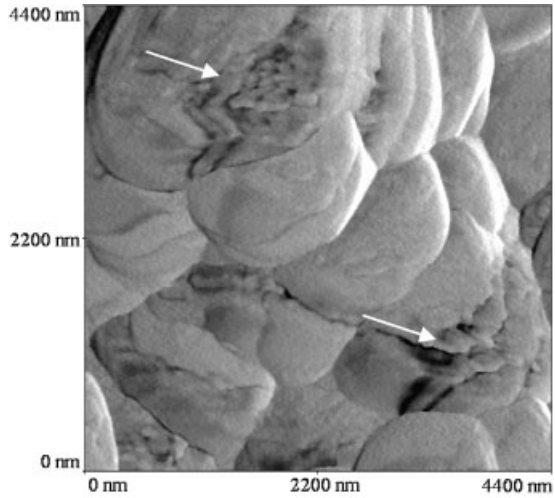

Figure 3.
AFM phase contrast image of the surface of a 120 min-treated PHB film.

cell morphology was analyzed qualitatively by scanning electron microscopy (SEM) after 7 days (Figure 4).

In vitro experiments indicated that the surface modification by aminolysis does not have toxic effects on the cultured cells, since they could adhere and proliferate on treated PHB films. Chondrocytes could adhere to the untreated PHB film surface, but numerous cells did not spread completely (Figure 4a). More adhered cells were observed on the surface of 45 min-treated films, but only few cells could spread completely when compared to untreated films (Figure 4b). It was observed that longer treatment time improved capability of PHB films to support cell growth (Figure 4c–d). The chondrocytes spread well at the surface of PHB films treated for 90 min and 120 min comparing to untreated films. In these cases, flatten shaped cells were visualized. The fibroblast-like morphology of the chondrocytes, that are naturally round-shaped in hyaline cartilage, was lost because chondrocytes when cultured in a monolayer de-differentiate into fibroblast.[15]

Table 1.
Root mean square (RMS) roughness of untreated and treated PHB films.

PHB Films	RMS Roughness (nm)
Untreated film	(92 ± 8)
45 min-treated film	(117 ± 15)
90 min-treated film	(112 ± 9)
120 min-treated film	(120 ± 16)

Conclusions

The chondrocytes adhesion to PHB films was successful enhanced after surface modification of these films via aminolysis with 0.1 M ethylenediamine. This wet-chemical method induced incorporation of amino groups onto the PHB films surface without any harmful effects to the cultured cells. According to the results, it is reasonable to suppose that the treatment with ethylenediamine might have increased the adsorption of adhesion molecules produced by own cells, propitiating differences in cell adhesion, spreading and proliferation on PHB films surfaces. The explanation for this seems to be mainly related to physico-chemical effect, since the surface charge of treated films was altered due to positively charged NH_2 groups introduced onto the surface. The topographical effects should also be taken into account, since it was observed a slight increasing in roughness of aminolyzed films. Further experiments will

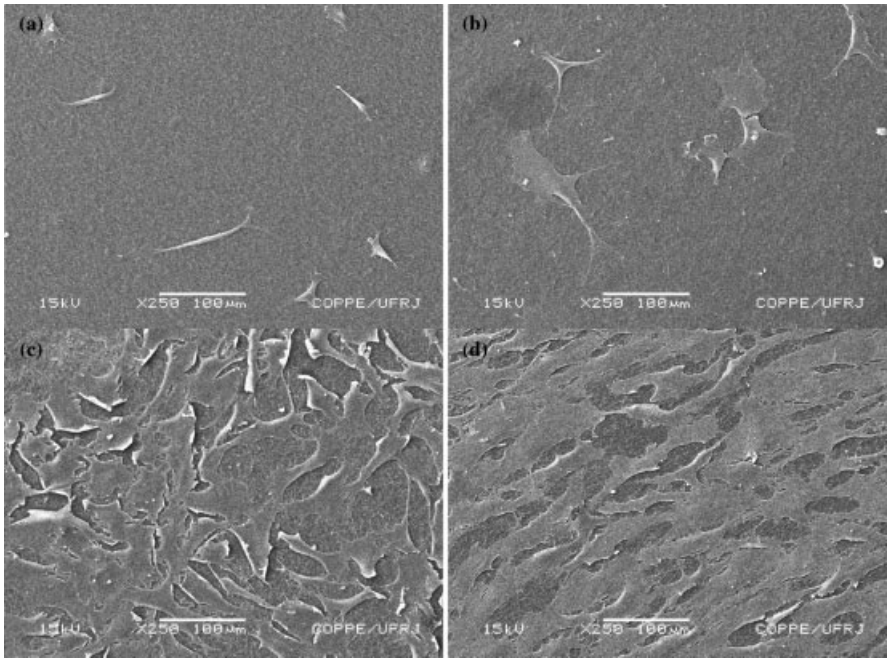

Figure 4.
SEM images of chondrocytes cultured on PHB films. Cells were cultured for 7 days on (a) untreated films and on aminolyzed films for (b) 45 min, (c) 90 min and (d) 120 min.

be conducted with PHB in a three-dimensional scaffold in order to evaluate the maintenance of the cartilage phenotype.

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