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POLY(L-LACTIC ACID) MEMBRANES: ABSCENSE OF GENOTOXIC HAZARD AND POTENTIAL FOR DRUG DELIVERY

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Abstract

Biomaterial is defined as any substance from natural sources or synthetic, that can be used in biological tissues without causing deleterious reactions to the body. Widely used for more than four decades, the titanium has an important role in various areas of health. Its use cannot be considered completely safe. The literature reports the presence of atrophy or bone deformation when this material is used for the treatment of fractures or rehabilitation in young patients, in addition to interfering in some imaging tests and uncomfortable to touch. The absorbable polymers, in particular the PLA, are materials with the advantage of being completely absorbed by the body. Although the use of PLA as material for bone synthesis is known, research with the purpose of better understanding the behavior of this polymer front to biological tissue are necessary, because it has different physical and chemical characteristics of titanium, so that you can offer advantages in relation to the metal. These in vitro studies evaluated the behavior of titanium and the PLA at the cellular level and molecular. No cytotoxic effect or genotoxic was observed in the tests with the cells 3T3 and CHO-K1. In addition to replication and gene expression remained unchanged in both experiments with titanium as with the PLA. The latter also showed the ability to incorporate and release molecules.

Keywords: Poly(l-Lactic acid); Citotoxicity, qPCR real time, Drug delivery

Resumo

Define-se biomaterial como qualquer substância proveniente de fontes naturais ou sintéticas, que possam ser utilizados nos tecidos biológicos sem provocar reações deletérias ao sistema. Amplamente utilizado a mais de quatro décadas, o titânio tem um papel importante nas diversas áreas da saúde. Ainda assim seu uso não pode ser considerado completamente seguro. A literatura relata a presença de atrofia ou deformação óssea quando este material é utilizado para o tratamento de fraturas ou reabilitações em pacientes jovens, além de interferir em alguns exames de imagem e apresentar desconforto ao toque. Os polímeros absorvíveis, em particular o PLA, são materiais com a vantagem de ser completamente absorvidos pelo organismo. Embora o uso do PLA como material para síntese óssea seja conhecido, pesquisas com a finalidade de compreender melhor o comportamento deste polímero frente aos tecido biológicos são necessárias, pois ele apresenta características físicas e químicas diferentes do titânio, de forma que pode oferecer vantagens em relação ao metal. Estes estudos *in vitro* avaliaram o comportamento do titânio e do PLA em nível celular e molecular. Nenhum efeito citotóxico ou genotóxico foi observado nos ensaios realizados com as células 3T3 e CHO-K1. Além disso a replicação e a expressão gênica se mantiveram inalteradas tanto nos experimentos realizados com o titânio como com o PLA. Este último ainda mostrou a capacidade de incorporar e liberar moléculas.

Palavras-chave: Ácido Poli(l-Láctico); Citotoxicidade; qPCR em tempo real, liberação de medicamentos.

Introdução geral

A busca por materiais com aplicabilidade clínica em cirurgias de reabilitação e reconstrução óssea é de grande importância uma vez que a grande maioria dos equipamentos utilizados no momento para estes fins, como por exemplo, o titânio, apresentam limitações quanto aos processos de absorção e osteoindução. Os efeitos do titânio quando utilizados em indivíduos jovens, em fase de crescimento, tais como a sensibilidade tátil e/ou térmica e risco de infecção e exposição ou até mesmo a deformação ou atrofia do osso fomentam os esforços da engenharia tecidual e biotecnologia em busca de novos biomateriais, que além de não oferecer risco durante a fase de cicatrização, possa, inclusive, acelerar os processos regeneradores.

Neste contexto os polímeros derivados de ácido poli-L-láctico (PLA) têm despertado grande interesse dos pesquisadores pelos seus efeitos biológicos e promissores devido à capacidade de serem reabsorvidos. Como resultado observa-se pouco ou nenhum efeito residual do material no local, e consequentemente menor inflamação. Além disso a possibilidade de incorporar moléculas talvez seja a principal vantagem sobre muitos biomateriais presentes no mercado.

A proposta dos estudos realizados vem de encontro com este panorama. Foram realizados testes que avaliaram o comportamento celular frente ao titânio e ao ácido poli-L-láctico, e que ajudaram a compreender alguns aspectos desta relação entre a célula e o biomaterial.

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Poly(L-lactic acid) membranes: Absence of genotoxic hazard and potential for drug delivery

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HIGHLIGHTS

- PLA (poly[lactic acid]) membranes do not exhibit cytotoxic and genotoxic effecs when tested in mammalian cells.
- Cell culture conditions do not affect the physicochemical properties of PLA membranes.
- PLA membranes exhibit potential for drug delivery systems.

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ABSTRACT

The use of poly(L-lactic acid) (PLA) has been considered an important alternative for medical devices once this polyester presents biomechanical, optical and biodegradable properties. Moreover, the use of PLA results in less inflammatory reactions and more recently it has been proposed its application in drug delivery systems. Genotoxicological evaluations are considered part of the battery assays in toxicological analysis. Considering the wide applications of PLA, the present work evaluated the potential cytotoxic and genotoxic effects of PLA in CHO-K1 cells, as well as its physicochemical properties. No cytotoxic effects of PLA were detected by colorimetric tetrazolium assay (XTT) analysis, and the clonogenic survival assay showed that PLA did not disrupt the replicative cell homeostasis, neither exhibited genotoxic effects as evidenced by comet and micronucleus assays. Thermogravimetric properties of PLA were not altered after contact with cells and this film exhibited ability in absorb and release Europium(III) complex. All these data suggest genotoxicological safety of PLA for further applications in drug delivery systems.

1. Introduction

Poly(L-lactic acid) (PLA) is a linear aliphatic polyester made from lactic acid monomers, ultimately made from lactose (or milk sugar) derived from renewable plant sources, such as starch and sugar (Silverajah et al., 2012). According to Huang et al. (1990) the development of materials such as PLA that are naturally synthesized using bacteria grown by fermentation, can be considered as truly biodegradable and eco-friendly after composting. The building block of PLA is lactic acid (2-hydroxypropionic acid) which can exist as optically active D- or L-enantiomers (Lim et al., 2008). The biomechanical, optical and biodegradable properties of PLA resulted in an important alternative for titanium use in medical devices once this biomaterial is bioabsorbable (Puleo and Nanci, 1999; Silverajah et al., 2012). Polymers such as PLA are degraded by simple hydrolysis of the ester bonds, which does not require the presence of enzymes and in turn prevents inflammatory reactions. The hydrolytic products from such degradation process are then transformed into non-toxic sub-products that are eliminated through normal cellular activity and urine (Lasprilla et al., 2012). More recently, PLA has been used for the development of drug delivery systems because of its excellent biocompatibility (Immich et al., 2013; Santos et al., 2013).

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During biocompatibility analysis of biomaterials, a great number of assays are required to ensure the chemical stability in the biological test system (Naaman et al., 2007). According to the International Organization of Standardization (ISO 10,993-1/EM 30,993-1) (ISO, 2003) toxic hazard and biological safety need to be evaluated following well established guidelines for genotoxicity assessment. Genotoxic analysis is important because the majority of recognized human carcinogenic substances are also genotoxic (Pfuhler et al., 2007).

In the present study the PLA film substrate was tested for cytotoxicity and genotoxicity using Chinese Hamster Ovary (CHO-K1) cell line. Cytotoxicity was tested in terms of cell viability and proliferation. The extension of primary DNA damage and further clastogenic or aneugenic effects were evaluated by comet and micronucleus assays, respectively. The physicochemical, thermal and photoluminescence properties of PLA films were also evaluated before and after the contact with cells as well as its ability to absorb and release chemical substances [Europium(III) complex] for further pharmaceutical purposes.

2. Materials and methods

2.1. PLA film substrate

Sterile PLA film substrates of 0.25 cm^2 were purchased from NeoOrtho (Curitiba, PR, Brazil) and kept under sterilized conditions. All manipulations were inside a laminar flow cabinet.

2.2. Cell culture conditions

CHO-K1 cells were cultured in complete medium composed of DMEM + HAM F10 (1:1, v/v) (Sigma, St Louis, USA) supplemented with 10% fetal calf serum (Sigma, St Louis, USA) and 1% of antibiotics penicillin/streptomycin stabilized solution (Sigma, St Louis, USA) in cell culture flasks of 25 mm^2 (TPP, Swizerland). Cells were incubated at 37 °C and 5% of CO_2 in humidified atmosphere. Cells were used between the 3rd and 8th passage and exposure to PLA membranes was according the recommendation of ISO 10,993-1/EM 30,993-1 (ISO, 2003).

2.3. Cell viability

Cell viability was determined using the Cell Proliferation Kit II (Roche Applied Science)

. This method is based on the principle of the cleavage of yellow tetrazolium salt XTT by metabolically active cells forming an orange formazan dye. Thus, this conversion occurs only in viable cells. For this assay 2×10^4 cells were seeded in 12-well plates containing complete medium and exposed to PLA films for 24, 48 and 72 h. Positive control (PC) was treated with 0.5 mM of Doxorubicin (Sigma, St Louis, USA) and negative control (NC) did not receive treatment. After exposure culture medium was removed and cells were washed with phosphate buffer solution, including positive and negative controls. Afterwards, cells were incubated with DMEM without phenol red containing the XTT/electron solution (50:1, v/v) that remained in culture for 4 h. Following, the volume of each well was transferred to ELISA microplate for colorimetric reading in a spectrophotometer (Tecan A-5082, Salzburg, Austria). The result of the absorbance measured at 492 and 690 nm (reference) is directly proportional to the number of viable cells in each treatment after 24 h post-exposure. Percentage of viable cells is expressed in proportion of the negative control considered with 100% of cell viability.

2.4. Clonogenic survival assay

The clonogenic survival assay is based on the principle that a single cell can grow to form a colony. For this assay 2×10^4 cells were seeded in 12-well plates and exposed to PLA films with the same conditions mentioned above. PC was treated with 0.25 mM of Doxorubicin (Sigma, St Louis, USA) and NC did not receive treatment. After 24 h cells were washed with phosphate buffer saline and trypsinized. Cell counting was performed in a Neubauer[1] chamber and 150 cells were seeded in 6-well plates containing complete medium. Cells were incubated at 37 °C and 5% of CO_2 for 7 days when colonies were visible.

The colonies formed were fixed with methanol/acetic acid/water (1:1:8 v/v/v) and stained with Giemsa 5% in Sörensen phosphate buffer (pH 6.8). The colonies were counted, and the cell surviving fraction was calculated as percent colonies relative to untreated controls (NC).

2.5. Comet assay

For comet assay 2×10^4 cells were seeded in 12-well plates and exposed to PLA films with the same conditions mentioned above. Positive control was treated with 0.25 mM of Doxorubicin (Sigma, St Louis, USA) and negative control did not receive treatment. After 24 h cells were washed with phosphate buffer saline and trypsinized. Then, 20 mL of cell suspension were homogeneized with low melting point agarose (0.5%) and layed onto a microscope slide pre-coated with standard agarose (1.5%). The material was covered with a coverslip and set on ice for 5 min when the coverslip was removed and slides was submerged in a cold lysis solution (NaCl 2.5 M, Na_2EDTA 100 mM, Tris 10 mM, Triton X-100 1% and DMSO 10%, pH 10) overnight. After that, slides were washed in a phosphate buffer and incubated in alkaline buffer solution (NaOH 0.3 M and Na_2EDTA 1 mM, pH > 13) for 20 min. Electrophoresis was performed in the same alkaline buffer at 25 V and 300 mA for 20 min. After electrophoresis, the slides were gently immersed in neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min and then fixed with ethanol. During comet analysis, slides were stained with ethidium bromide solution (20 $\mu g\ mL^{-1}$) and nucleoids visualized in a Axio Imager fluorescence microscope (Carl Zeiss, Germany) equipped with a 515–560-nm excitation filter and a 590-nm barrier filter at 400X magnification. The % of DNA in tail was considered as the result of DNA damage extension.

2.6. Cytokinesis-blocked micronucleus (CBMN) assay

CBMN assay followed the recommendations of Fenech (2000) with minor adjustments. Briefly, 2×10^4 cells were seeded in 12-well plates and exposed to PLA films with the same conditions mentioned above. Positive control was treated with 0.25 mM of Doxorubicin (Sigma, St Louis, USA) and negative control did not receive treatment. After 24 h of exposure, 5 $\mu g\ mL^{-1}$ of cytochala- sin-B (CytB) was added to the CHO-K1 cultures remaining for additional 24 h. Then, cells were trypsinized and treated with a cold lysis solution of sodium citrate 1% (w/v) for 3 min. The cells were fixed two times with methanol:glacial acetic acid (3:1,v/v) and the cell suspensions were dripped on a slide. Giemsa solution 5% diluted in Sorensen phosphate buffer (Na_2HPO_4 0.06 M, KH_2PO_4 0.06 M, pH 6.8) was used for slide staining. One thousand cells were scored in a conventional light microscopy (400X magnification) to evaluate the percentage of mono-, bi-, tri-, and tetra-nucleated cells. The nuclear division index (NDI) was calculated according to the formula: NDI = M1 + 2(M2) + 3(M3) + 4 (M4)/N, where M1–M4 represents the number of cells with 1–4 nuclei, respectively, and N is the total number of scored cells. Micronuclei

(MN) were scored in 1000 binucleated cells according to the criteria of Fenech (2000).

2.7. Characterization of PLA films before after cell culture

Thermal analysis was conducted using the Thermal Analyst 2100–TA Instruments SDT 2960 simultaneous DTA-TG in nitrogen atmosphere, at a heating rate of 20 °C/min, from 25 °C to 900 °C.

PLA films were exposed to 0.1 mol L^{-1} Europium(III) complex with dipicolinic acid ($Na_3[Eu(dpa)_3]$), a high luminescent compound. The photoluminescence properties of the complex were studied by the excitation and emission spectra which were conducted on a Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter equipped with a continuous xenon lamp (450 W) as the radiation source, a SPEX double monochromator model 1680, and a photomultiplier R 928 Hammatsu. The measurements were collected at 90° with respect to the incident beam. The excitation (f_{exc}) and emission (f_{em}) slits were 2.0 and 1.0 nm.

2.8. Statistical analysis

All analyses were performed considering three independent experiments in duplicate. Results were expressed as mean ± SE and compared using one-way analysis of variance followed by Tukey's test ($p < 0.05$) using the GraphPad Prism™ (Version 4.00) software (GraphPad Software, Inc.,-San Diego, California, USA).

3. Results and discussion

3.1. Evaluation of cytotoxicity

Cytotoxicity of PLA films was evaluated by the XTT cell viability assay and clonogenic survival. Results of cell viability are represented in Fig. 1. Exposure to PLA in different times did not result in modification of cell viability in comparison to the negative control (NC) ($p < 0.05$). The effect of PLA on cell replication was evaluated by clonogenic survival assay. Results obtained demonstrated a slight, but not significant, increasing in the fraction of survival cells (112.3%, $p = 0.062$) compared to negative control (100%) (Fig. 2). Literature reports the evaluation of cytotoxicity by different approaches, including the MTT and XTT methodologies that screening cell viability based on the fraction of metabolic active cells. In XTT assay the sodium 2,3-bis(2-methoxy-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt is reduced by the mitochondrial reductase enzymes resulting in the water soluble formazan (Ishyiama et al., 1996; Scudiero et al., 1998). To ensure feasibility of XTT assay doxorubicin was used as PC at 0.5 mM. Exposure of CHO-K1 cell line do PLA film resulted in a non-significant reduction of 4.8% of cell viability in comparison to NC. The method of cell exposure when biomaterials are under

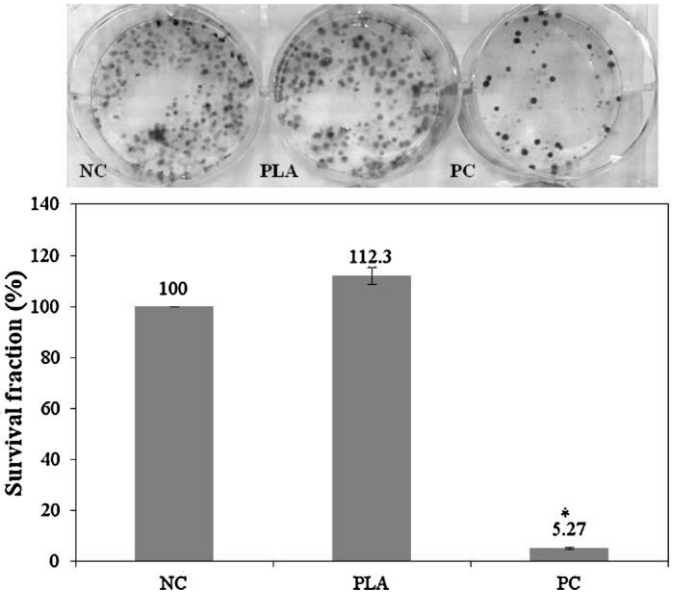

Fig. 2. Survival fraction of CHO-K1 cells seven days after 24 h of exposure to PLA film. NC: negative control; PC: positive control (DXR 0.25 mM). *Statistically significant with respect to negative control $P < 0.05$.

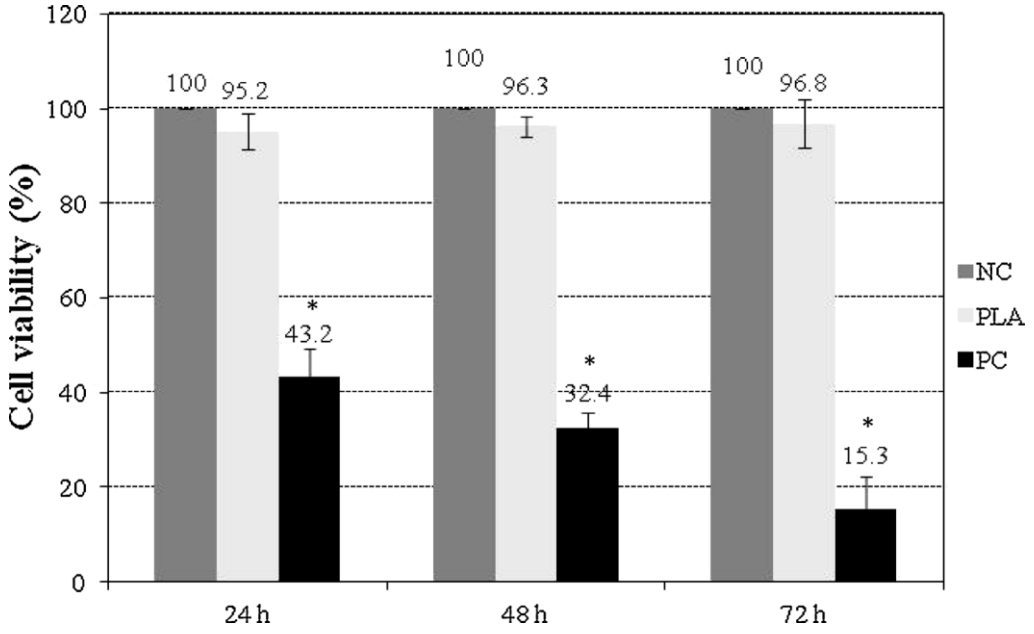

Fig. 1. Mean values of cell viability obtained by XTT assay in CHO-K1 cell line after 24 h, 48 h and 72 h exposure to PLA film. NC: negative control; PC: positive control (DXR 0.5 mM). Three independent experiments were performed. *Statistically significant with respect to negative control $P < 0.05$.

toxicological evaluation has been discussed in the literature (Inayat-Hussain and Rajab, 2008). ISO 10,993-3 recommendation (ISO, 2003) is that cell treatment or exposure may preferentially attend the biomaterial form employed in clinical practice, including the *in vitro* approach. The direct contact test has the advantage that it (i) mimics the physiological conditions, (ii) has a zone of diffusion (a concentration gradient of toxic chemicals), and (iii) affects cellular trauma in accordance with the density of materials (Ratner and Northup, 1996; Baek et al., 2005).

Results obtained by the clonogenic survival demonstrated that replicative properties of CHO-K1 cells were preserved after exposure to PLA membranes. There was a slight increase in survival fraction of cell cultures exposed to PLA membranes. According to Sumantran et al. (2007), the clonogenic survival, or cell colony forming assay, evaluates the ability of cells to maintain the replicative capacity revealed by the presence of cell colonies. Therefore, for this assay, PC was treated with 0.25 mM of doxorubicin, once cells treated with this concentration maintain survival but is unable to replicate, as determined in preliminary experiments (data not shown). Clonogenic survival assay is a parameter of cell survival representing a long term cytotoxicity evaluation method. Taken together, these data suggest that cellular viability and replicative properties are preserved in CHO-K1 cells after exposure to PLA film.

3.2. Genotoxicological analysis

The genotoxicity of PLA film was evaluated in CHO-K1 cells as recommended before (Yalkinoglu et al., 1990). According to the Organization for Economic Co-operation and Development (OECD, 2014) the micronucleus assay is robust and effective in a variety of cell types, including CHO cells, in the presence or absence of cytochalasin B. Genotoxic analysis was conducted using comet and CBMN assays and results obtained are exhibited in Table 1. The extension of DNA damage detected by comet assay is represented by the percentage of DNA in tail. Exposure to PLA membranes did not increase the % of DNA in tail (13.7 ± 0.3) in comparison to the negative control (13.2 ± 2.4) ($P > 0.05$) neither the mean of binucleated cells with micronucleus (BNMC) (5.6 ± 2.5 in PLA group *versus* 4.4 ± 1.5 in negative control, $P > 0.05$). Nuclear division index (NDI) was not affected by PLA exposure ($P > 0.05$).

The L-isomer of poly lactic acid has gained great attention because of its excellent biocompatibility, however its long degradation times coupled with the high crystallinity of its fragments can cause inflammatory reactions in the body (Lasprilla et al., 2012). In order to overcome this, PLA can be used as a material combination of L-lactic and D, L-lactic acid monomers, being the latter rapidly degraded without formation of crystalline fragments during this process (Fukushima and Kimura, 2008). The genotoxicity of poly(D/L-lactic acid), was evaluated 90 and 120 days after implantation of PLA disks in the calvarium in contact with the underlying dura mater and overlying periosteum of male rats (*Rattus novergicus*). Results from micronucleus test indicated absence of cytotoxicity and genotoxicity in bone marrow erythrocytes after long exposure to PLA implant in calvarium (Göelzer et al., 2012).

There is extensive use of PLA membranes for bone repair in surgery processes (Charbit et al., 1999; Javed et al., 2012). Considering our results, 24 h of exposure to PLA film did not result in genotoxic effects to CHO-K1 cells. Comet and micronucleus assays have been used in genotoxicity screening of biomaterials disks (Saska et al., 2012). Exposure to PLA did not increase the extension of DNA damage expressed by tail intensity in comet assay. Primary DNA lesions detected by comet assay can be efficiently repaired; otherwise, they can give rise to stable mutations. Results obtained from micronucleus test confirmed the data obtained by comet assay. Exposure to PLA membranes did not increase the frequency of MN in binucleated cells. It is well known that MN is expressed in cells when either acentric chromosome fragments of whole chromosomes fail to be segregated to the daughter nuclei during mitosis (Fenech and Bonassi, 2011). Therefore, in conditions of exposure to clastogenic and/or aneugenic toxicants frequencies of MN are increased and genotoxicity is confirmed.

The genotoxicity of PLA copolymers has been reported. The co-polymerization of PLA with glycolic acid results in the copolymer poly(lactic-co-glycolic acid) (PLGA). The fraction of glycolic acid during co-polymerization affects the rate of degradation and ability to cell attachment of PLA-based membranes (Fialho et al., 2003). Human articular chondrocytes exhibited better attachment and proliferation on PLA than PLGA surfaces (Ishaug-Riley et al., 1999). Therefore, the genotoxicity of PLGA copolymer nanoparticles was investigated by Kazimirova et al. (2012) using comet and CBMN assays in TK6 lymphoblastoid cell line. Results with comet assay showed that exposure to PLGA-PEO nanoparticles for 2 or 24 h induced neither strand-breaks nor oxidized DNA lesions, suggesting that PLGA-PEO nanoparticles are not potentially genotoxic. They also investigated the genotoxicity of PLGA-PEO in binucleated (MNBNCs) and mononucleated (MNMNCs) cells obtained by CBMN assay using two protocols: the addition of cytochalasin B during the final 24 h of treatment, but with the nanomaterial present for the duration of cytochalasin B treatment, and the addition of cytochalasin B after removal of the nanomaterial medium. There were no differences in the frequency of MNBNCs between negative control and cells treated with PLGA-PEO, suggesting that these nanoparticles are not a potential genotoxin. However, the analysis of MNMNCs revealed an increased frequency of mononucleated cells with micronucleus after 48 h treatments with PLGA-PEO when cytochalasin was added for the last 24 h, and for a 24 h treatment with PLGA-PEO nanopaticles followed by washing off the particles and the adding cytochalasin B for further 24 h, but these results were inconclusive regarding a possible weak aneugenic potential of PLGA-PEO nanoparticles or an early genotoxic effect (Kazimirova et al., 2012). On the other hand, no genotoxic effects of PLGA nanoparticles were detected in human skin fibroblast cell line BJ at molecular level as demonstrated by the expression of γH2AX (Setyawati et al., 2013).

While the presence of glycolic acid in the lactide PLGA copolymer increase the hydrophilic properties and hence reduces the tissue absorption compared to PLA (Makadia and Siegel, 2011), the last one demonstrates the ability to enhance osteoblastic activity and the formation of collagen type I in skeletal stem cells (Tayton et al., 2014). Moreover, PLA stabilizes bone lesions more efficiently than PLGA and contributes more efficiently to vascular repair tissue of meniscus (Klompmaker et al., 1991). On the contrary, PLGA is more suitable for drug delivery than PLA (Abdollahi and Lotfipour, 2012). Therefore, considering we tested PLA in the form of membranes used for tissue

Table 1
Mean ± standard deviation (M ± SD) of tail intensity, binucleated cells with micronucleus (BNMC) and nuclear division index (NDI) obtained by comet and micronucleus assay in CHO-K1 cells exposed for 24 h to PLA film.

Group	Tail intensity mean±SD	BNMC mean±SD	NDI mean±SD
Negative control	13.2 ± 2.4	4.4 ± 1.5	1.6 ± 0.02
PLA	13.7 ± 0.8	5.6 ± 2.5	1.7 ± 0.01
Positive control	317.2 ± 0.9*	26.6 ± 4.5*	1.6 ± 0.03

* $P < 0.05$ with respect to negative control.

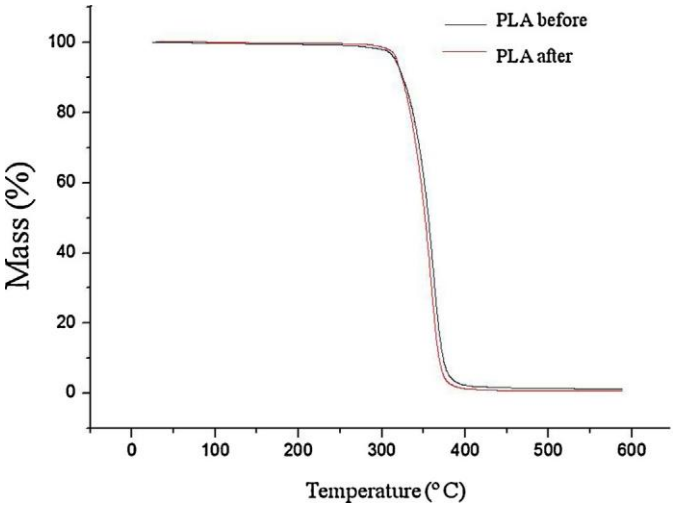

Fig. 3. Thermogravimetric curve of PLA film before and after contact with CHO-K1 cell. The temperature of thermal decomposition is between 300 and 400 °C.

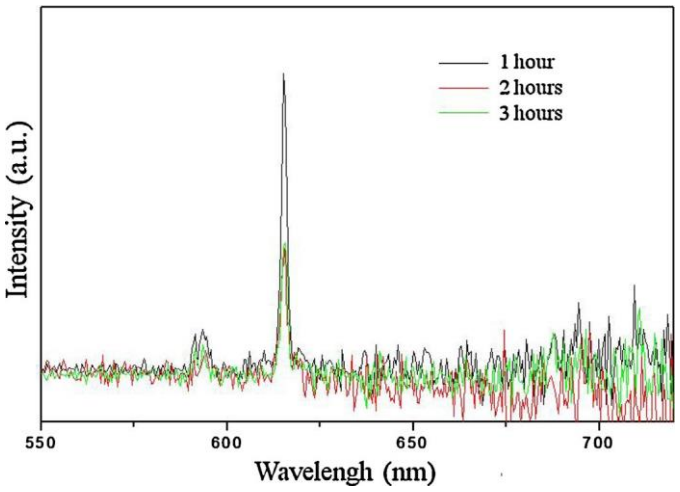

Fig. 5. Emission spectrum obtained in Europium-incorporated PLA films 1, 2 and 3 h after ethanol elution. a.u.: arbitrary units.

repair, we tested its physico-chemical properties and ability for drug delivery.

3.3. Physical and chemical characterization

After genotoxicity evaluation we tested the chemical and thermal properties of PLA before and after contact with CHO-K1 cells. The physical and chemical characterization of PLA films before and after 24 h in contact with CHO-K1 cell line is represented in Fig. 3. The thermogravimetric curve shows that previous contact with cells did not induce alteration in PLA membranes, and that thermal decomposition occurs in temperatures from 300 to 400 °C before and after contact with CHO-K1 cells. These results are confirmed by the infrared spectroscopy where no structural alterations were detected before or after contact with cells. Temperature of thermal decomposition of PLA membranes was the same in exposed and non-exposed membranes to cell contact. Likewise, the contact of PLA film for 24 h with CHO-K1 cells did not result in structural alterations on film surface (Fig. 3), demonstrating that exposure of PLA to cell culture conditions did not affect its thermal and structural properties. Physico-chemical properties of L-PLA form was tested in comparison with D-L forms by Engelberg and Kohn (1991) that demonstrated strength of L-PLA increased compared to D,L-PLA. The authors suggested that L-PLA is clearly the preferred polymer for high strength applications such as orthopedic implants.

PLA films were also tested for their ability to absorb or release chemical substances. The maximum excitation spectrum was at 273 nm, and resulted after Europium incorporation in PLA membranes before and after contact with CHO-K1 cells (Fig. 4). After Europium(III) complex incorporation, PLA films was kept in contact with ethanol and the luminescent intensity expressed in arbitrary units was evaluated 1, 2 and 3 h after. The spectrum of emission is exhibited in Fig. 5. We observed a reduction in luminescent emission from PLA films. According to calculated arbitrary units of emission intensity, the release of Europium complex was stabilized after 2 h in ethanol solution. Therefore, the percentage of Europim (III) in PLA membranes was significantly reduced 2 and 3 h after contact with ethanol solution. We previously observed the maximum excitation of Europium(III) at 273 nm when incorporated to acrylonitrile butadiene styrene polymer (de Souza et al., 2012) and hybrid materials (Azevedo et al., 2013). Therefore, the results observed in Fig. 4 confirm the ability of PLA membranes no incorporate Europium(III) complex.

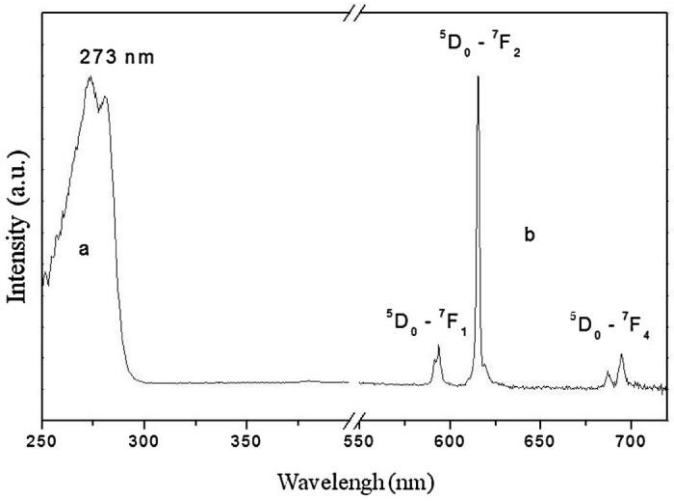

Fig. 4. Excitation (a) and emission (b) spectrum of Europium(III) complex after incorporation to PLA film. $^5D_0$–$^7F_1$, $^5D_0$–$^7F_2$ and $^5D_0$–$^7F_4$ represent the ion energy level related to electronic transitions occurring in Europium(III) compound. a.u.: arbitrary units.

The emission spectrum of the Eu^{3+} ion in the complex displays emission lines corresponding to transitions from the excited state $^5D_0$ to the fundamental level ^7F_J ($J = 0, 1, 2, 3$ and 4), dominated by the $^5D_0 \rightarrow {}^7F_2$ (rv614 nm) electric dipole transition that occurs in the red region of the electromagnetic spectrum. Such spectrum was detected after contact of PLA membranes and CHO-K1 cells, demonstrating that membrane porosity can accommodate Europium(III) complex. This result confirms the ability of PLA to incorporate molecules with pharmacological purposes such as anti-inflammatory drugs, antibiotics and chemotherapeutic agents.

4. Conclusions

In conclusion, exposure to PLA membranes did not result in cytotoxicity to CHO-K1 cell, the replicative properties were maintained and no genotoxic effects were detected. Moreover, chemical and thermal properties of PLA membranes were not altered as well as PLA exhibited ability to absorb and release molecules confirming its potential application in drug delivery systems.

Conflict of interest

The authors declare no conflict of interests.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Considerações finais

A relação que se faz entre os estudos realizados mostra que os resultados do comportamento dos fibroblastos expostos ao PLA são positivos. No estudo observou-se ausência de efeitos citotóxicos e genotóxicos. Os efeitos citotóxicos foram comprovados nos experimentos realizados no estudo. Através dos dados destes dois experimentos conflitamos e confirmamos os resultados do estudo anterior realizado por Göelzer[18], afirmando não haver efeitos citotóxicos e genotóxicos para células expostas ao PLA. A ausência destes efeitos permite concluir que o material pode ser seguro para uso em humanos. Ainda assim entender-se ser necessário comparar os resultados do PLA ao material de escolha atual: o titânio.

O número de estudo realizado com o titânio são exponencialmente maiores do que os estudos realizados com o PLA, portanto a comparação do comportamento celular entre estes dois tipos de biomateriais é válida e importante para se comparar os resultados dos testes ao qual são submetidos. Esta comparação, mesmo em ambiente *in vitro,* pode fornecer dados importantes que permitem uma previsão de comportamento celular em ambiente *in vivo* já exaustivamente realizados com o titânio, e com eficácia comprovada. O titânio atualmente é o biomaterial de preferência para a utilização em seres humanos. No estudo pôde-se observar que não há diferenças estatisticamente significantes entre a expressão gênica dos fibroblastos em contato com o PLA e com o Titânio. Este dado mostra que o PLA além de ser seguro para utilização, não apresenta desvantagens de utilização em relação ao titânio tanto em nível celular quanto molecular. Embora não se tenha observado efeitos indutores do PLA ou do Titânio no ciclo celular nem mesmo na expressão gênica das células, foi possível observar ausência de efeitos nocivos na sobrevida e proliferação celular. Este dado pode ser considerado positivo, pois apesar de não se poder atribuir ao PLA a

capacidade de produzir efeitos indutores, os ensaios de caracterização física e química do estudo mostraram a capacidade do polímero de absorver e liberar moléculas, inclusive a capacidade de liberação controlada. Esta característica do material abre caminho para novos estudos no campo da engenharia biomolecular, pois precede, de forma muito positiva, novos estudos no intuito de se incorporar moléculas que possam induzir a proliferação celular. Além disso, substâncias poderão futuramente ser incorporadas ao polímero, tais como antinflamatórios e antimicrobianos, fruto de estudos quem poderão ser realizados no campo da engenharia farmacêutica.

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