Effects of irradiated biodegradable polymer in endothelial cell monolayer formation

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Abstract

In this work we study cell adhesion, proliferation and cell morphology of endothelial cell cultured on poly-L-lactide acid (PLLA) modified by heavy ion irradiation. Thin films of PLLA samples were irradiated with sulfur (S) at energies of 75 MeV and gold (Au) at 18 MeV ion-beams. Ion beams were provided by the Tandar (Buenos Aires, Argentina) and Tandetron (Porto Alegre, Brazil) accelerators, respectively. The growth of a monolayer of bovine aortic endothelial cells (BAEC) onto unirradiated and irradiated surfaces has been studied by in vitro techniques in static culture. Cell viability and proliferation increased on modified substrates. But the results on unirradiated samples, indicate cell death (necrosis/apoptosis) with the consequent decrease in proliferation. We analyzed the correlation between irradiation parameters and cell metabolism and morphology.

Key words: PLLA, Irradiated polymers, Swift heavy ions, Cell adhesion, Cell proliferation.
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1. Introduction

Vascular endothelium is a simple squamous epithelium which lines the inner surface of blood vessels facilitating laminar flow of blood and preventing the adhesion of

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blood cells. This epithelium is not an inert barrier without a biological relevance, is an active metabolic component that performs multiple physiological functions such as control of vascular tone and permeability of blood vessels [1]. Alteration of these functions causes dysfunction of both organs and tissues with the subsequent development of disease. Large numbers of patients suffer from diseases of the vascular system, resulting in a clinical need for developing functional arterial replacements [2]. In vitro models of endothelial cells represent a valuable alternative to study the behavior of the endothelium to different physiological stimuli and pathological aggression [3].

An essential requirement for culturing cells in vitro is a surface that provides a firm attachment of anchorage-dependent cells under in vitro culture conditions. Further cellular processes such as proliferation, survival, differentiation, and migration are driven by the polymer surface characteristics [4, 5]. For this reason in many cases the polymer surface must be modified to achieve specific adhesion and therefore the bioactivity properties change [6, 7].

Poly-L-lactide acid (PLLA) is a biodegradable polymer used as drug delivery systems and bioabsorbable sutures [8]. However, the limitation of aliphatic polyesters is their limited affinity to plasma membrane receptors and macromolecules which act as mediators of cell adhesion on a substrate [9]. It is difficult to culture isolated cells in PLLA substrates because cell attachment is rather low due to its hydrophobic properties. The functionalization of the chemically inert PLLA surface emerges as a useful approach to solve this problem.

Diverse methods have been used such as: plasma treatment [10],photo-induced grafting polymerization [11] and chemical modification through the blending process have been used [12, 13]. But these chemically based approaches require multiple complicated steps. Ion beam irradiation is an alternative way to modify the physicochemical properties of material surface because of their relative simplicity. Our previous study [14] reported that after irradiation into a PLLA polymer, the surface topography changed and the contact angle was reduced, making a more hydrophilic surface. FTIR measurements showed that the material became amorphous after irradiation and the CO bands decreased according to XPS results. However, the ion irradiation applied does not change the degradation properties of the analyzed polymer [14].

Several investigations have been reported using low energy ion bombardment [15, 16] and in particular for PLLA [17]. Most of the previous works analyzed the correlation of physical and chemical parameters with the number of cells attached to the surface.

In the present works we analyzed, in additions to cell adhesion, its evolution as

a function of time. We measured the cell proliferation parameter and observed their morphology until its cover the total irradiated surface. Bovine Aortic Endothelial Cells (BAEC) were used as an anchorage-dependent model cell for mono layer formation test. In this work we compare the effects produce by two different beams, namely Au (18 MeV) and S (75 MeV). These ions beams deposit energy in concentrated surface spots, randomly distributed, with similar stopping power of about 22 MeV/cm^{-2} but with different ion velocity Au (0.09 MeV/amu) and S (2.34 MeV/amu). The ion velocity determined the size of the ion spot and then results a different deposited energy density. Recent work [18] establish that cells can sense objects of a few tens of nanometers in size like these ions spots. And if their spatial distribution, chemical compositions and physical properties are appropriated, they can attach and proliferate on this substrate. The purpose is to compare the results of this low and hight energy irradiations that deposited different energy density as a function of the spatial distributions of ion spots (fluence) by measuring the proliferation and morphology of the cells.

2. Experimental Procedure

In the following, we describe the irradiation procedure, the cultured cell on the irradiated polymers process and morphology, adhesion and spreading evaluations.

2.1. Polyester irradiation

PLLA polymer of commercial grade with a thickness of 50 μm was provided by Goodfellow (England) and used "as received". The samples were irradiated in a vacuum of 10^{-6} - 10^{-7} Pa with ion beams perpendicular to the surfaces. The sulphur heavy ions beam (75 MeV) were provided by the Tandar (Buenos Aires, Argentina) and the gold beam (18 MeV) by the Tandetron (Porto Alegre, Brazil) accelerators. The irradiation times for each sample were varied as a function of the current intensity and required ion fluences of 1 x 10^{10} to 6 x 10^{11} ions cm^{-2}. For the different irradiated samples the current density was between 0.25 to 1.50 nA cm^{-2}, to minimize sample heating. After irradiation, the samples were stored in air.

2.2. Cell culture and conditions

The endothelial cell line derived from the tunica intima of bovine aorta BAEC were routinely cultured in Dulbecco Modified Eagles Medium (DMEM GIBCO) high glucose supplemented with 20 % FBS (Fetal Serum Bovine, Natocor, Argentina) and 1 % streptomycin-penicillin antibiotic solution. Cells were incubated at 37 °C with 5 % CO_2/95 % of air and relative humidity of 95 % atmosphere. After treatment with

trypsine 0,25 %(Sigma) - 0,02 % EDTA in PBS, the cells were harvested. Then, the cell density was adjusted to 2 x 10^4 cells/mL and were seeded onto 24-wells TCPS covered with the studied polymers (1.8 x 10^4 cells cm^{-2}). Cells were used between passages 4 and 7 for all experiments.

Both ions irradiated and control pristine PLLA were sterilized prior to inoculation of the cells. The films sterilization process was carried out first by soaking in 70 % C_2H_5OH solution and then rinsed with deionized water and before being rinsed 3 times with 1X phosphate buffer (Phosphate buffered saline:PBS) and finally with Milli-Q water. Then, they were placed on the bottom of 24 wells TCPS and covered with a sterilized Viton® ring to prevent floating. The culture wells were incubated under the same experimental conditions.

2.3. EC adhesion and biochemical activity

The cells were seeded onto irradiated and unirradiated PLLA films and incubated for 1, 4, 6 and 7 days. After these periods of time, we monitored the viability and biochemical activity of cell populations. To this end, were used metabolic conversion of MTT (reduction of 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a purple formazan product). The cells were incubated with MTT (Sigma Chemicals, USA) during 2 h and the insoluble formazan product was solubilized with dimethyl sulfoxide solution (DMSO, Sigma Chemicals). The absorbance for formazan solutions obtained from the above-mentioned films was measured in a plate reader Beckman Coulter DTX 880 at 560 nm. The values obtained were measured using wells in equivalent position for a significative comparison.

2.4. Morphology, spreading and actin stress fibers evaluation

Morphology of endothelial cell cultured on the tested surface was then evaluated on microphotographs taken with an Olympus BX51 microscope (Obj. 100x and 40x). Components of the cytoskeleton can be imaged using fluorescent staining techniques. To visualize actin filament structure the attached cells were treated using a technique described in a previous work [19]. The specimens were examined using an Olympus BX51 microscope.

3. Results and discussion

The purpose of our work is to measure the number of cells attached to the irradiated surface as a function of different parameters such as: deposited energy, ion fluence and culturing times. Then we analyzed the range in which cell proliferation is maximized and cover the irradiated surface.

The cell quantity is measured, as described before, by detecting the absorbance of MTT which is proportional to the cell number. A normalized cell intensity is obtained from the ratio between the absorbance of the irradiated and the pristine sample. The final values are the average of three independent measurements and the statistical significance of the difference between experiments was evaluated by the Student t-test.

Figure 1 shows the percentage of the normalized cell number as a function of ion fluence and culturing times. As it can be seen the attachment increases with the culturing time. During the first 1-4 days of culturing the cells start to grow moderately and after 6-7 day of culturing a large increase was observed.

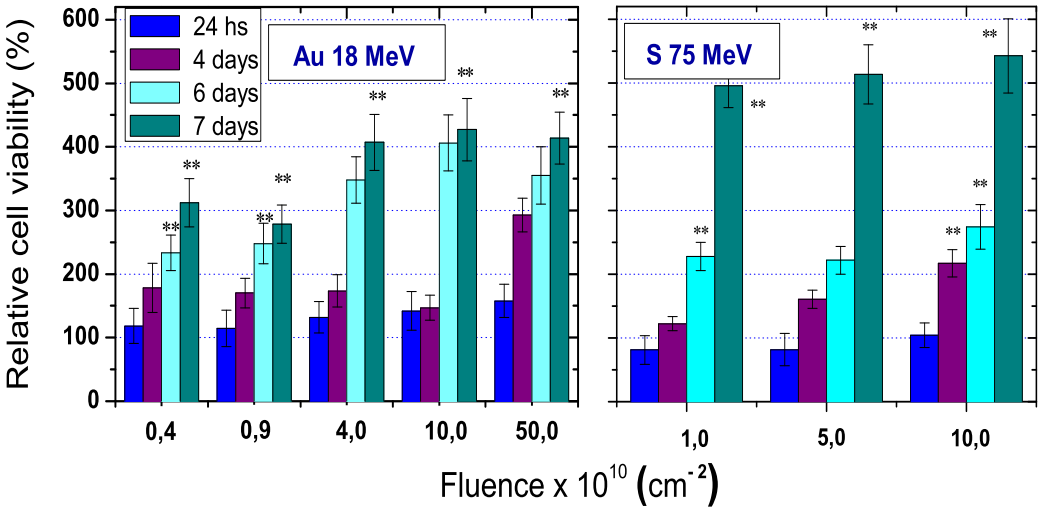

Figure 1: The cell attachment trends of BAEC cultured on PLLA supports by MTT-tetrazolium measurements. Groups of data points, that are significantly different compared to 4, 6 and 7 days on the control are indicated by (**): $p \leq 0{,}05$.

Figure 1 shows how the proliferation reaches a maximum value of four times (400 %) increase, for the sample irradiated with Au beam and with fluence more than 4 x 10^{10}. As it can be observed, in the analyzed ion fluence range of sulphur beam, an almost constant value of 500 % were measured.

With the purpose as analyzing the cell proliferation velocity at different culture times, Figure 2 shows the absorbance ratio between irradiated and pristine samples as a function of culture times. It shows a progressive increase in the rate of proliferation and a distinctive acceleration for sulphur beam treated samples cultured during 7 days.

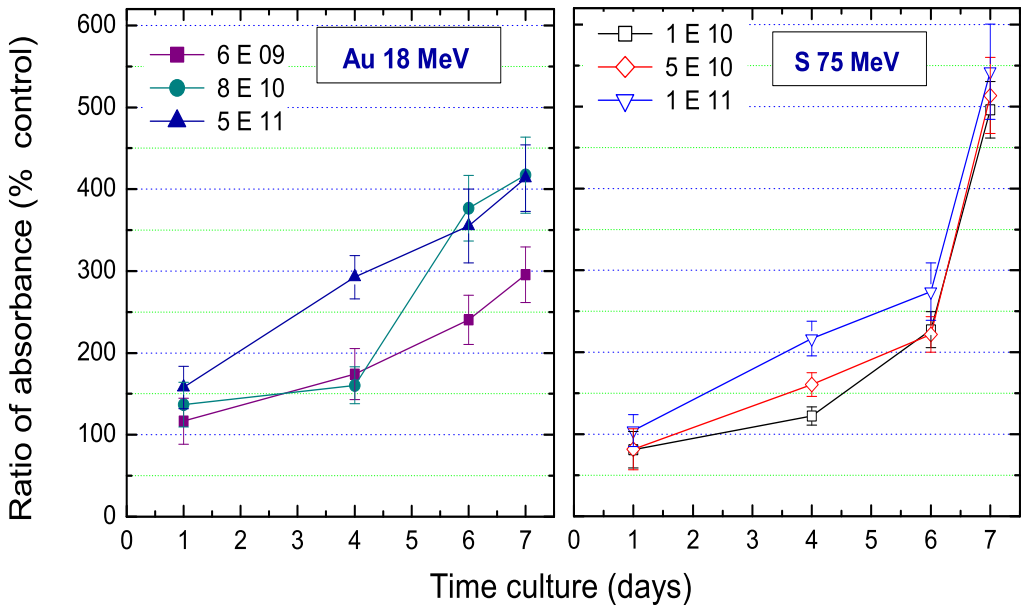

Figure 2: Enhanced proliferation for times profiles of cell adhesion in cultures of various fluences. Bars show standard deviations.

Figure 3 shows the overall cell morphology of BAEC cells adhering and proliferating on the surface observed with 100x and 40x objective. Figure 3 a) show the pristine surface with same groups of adhered cells that later produce a gradual decrease in metabolic activity which indicates cell death.

Figure 3 b) and c) show the results of the effects of different ion beams with the same 4 days cultured time. The films irradiated with Au beam (Figure 3 b) shows a more globular aspect compared with the compact distribution of cells in those irradiated with sulphur beam (Figure 3 c). Figure 3 d) shows the treated surface with the sulphur beam. After 7 days the proliferation produces the full coverage

of the available surface and the closed contact between this particular type of cells inhibits the proliferation process.

To determine the cells density for each sample, the mean value of the number found in twenty squares of 100 μm^2 size, were measured. The density of cells, after 7 days of culturing, for both Au and S beams resulted: 2.9 and 3.7 x 10^5 cells/cm^2 with an uncertainly of 20 %, respectively. Taking into account that the number of cells increased by a factor of 5 times after 7 days of culture (Figure 1) then it is possible to infer the initial numbers of adhered cells. The result of approximately 7 x 10^4 cells/cm^2 left about 80 % of the initial surface free of attached cells.

At the beginnig, the cells develop specific places, termed focal adhesions points, which allowed them to adhere to the surface and later, if the surrounding were appropriate, they started the proliferation process.

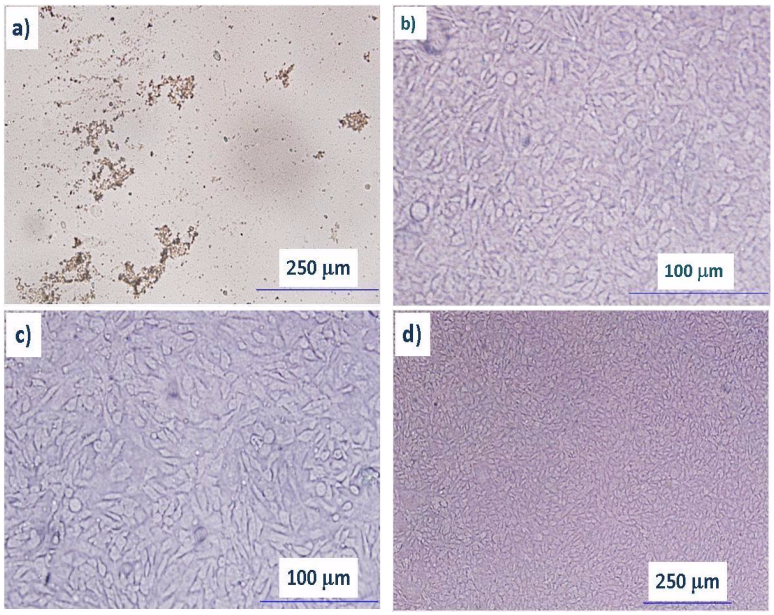

Figure 3: Phase contrast microphotographs of endothelial cells attached on PLLA for different energies and fluences: a) pristine: 4 days, b) Au, 18 MeV: subconfluent culture, 4 days; c) S, 75 MeV: subconfluent culture, 4 days; d) S, 75 MeV: cell monolayer, 7 days. See text for details.

In order to evaluate the cell structure, which is a crucial indicator of endothelial cell function, we used a fluorescence microscopy to observe the nuclei and changes in F-actin cytoskeleton. The cell adhesion sites depend on the stress fibers in the actin cytoskeleton for their creation and development. As it can be seen in Figure 4 the irradiated sample with sulphur beam shows cells with well-defined stress fibers and

the actin staining was intense. This indicated an intact network of actin filaments which is one of the crucial structures contributing to the morphological framework of a cell, and participates in the dynamic regulation of cellular functions. Moreover, an intact actin cytoskeleton as well as the contractility of the filaments facilitated by acto-myosin are required for the mobility of adhesions and also for components within focal complex. Furthermore, development of actin stress fibers was significantly improved on all irradiated surfaces after 4 days, where it is observed that the staining was enriched in the areas of cell-cell contact.

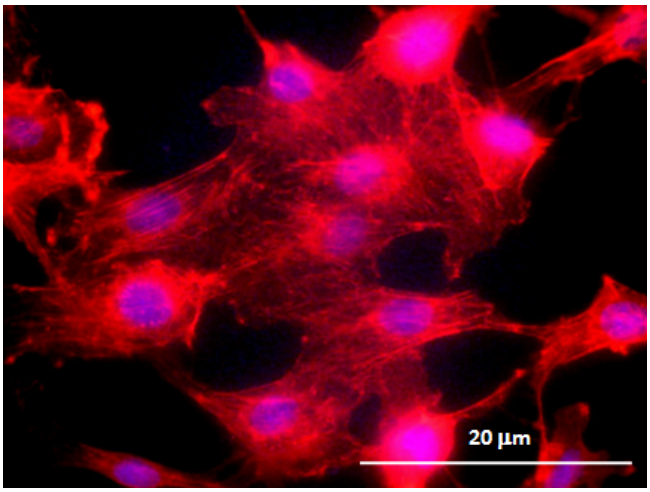

Figure 4: Distribution of actin filaments as a function of culture density. Fluorescence micrographs of spread BAEC cells stained for F actin by rhodamine- conjugated phalloidin (red) and the nucleus with DAPI (blue), S 75 MeV in subconfluent culture.

4. Conclusion

To our knowledge, the present work study for the first time the proliferation process on irradiated PLLA with heavy ion beams. Endothelial cell proliferation, morphology and mono layer formation on irradiated PLLA polymers was evaluated. The results indicate that BAEC is responsive to an irradiated substrate. From the results of biochemical activity, the number of adhering cells increased with fluence, in addition to inducing the desired cell morphology which is necessary for BAEC physiological function. The modification of the polymer surface is based mainly on the production of the new chemical groups induced by the ion deposited energy. The dissipation of ion energy is mediated by energetic electrons and the dose distribution

around the ion track has been the subject of several studies [20, 21]. The radius of the cylinder around the ion trajectory is determined by the range of the electrons. This range is proportional to the ion velocity [22] hence for about the same stopping power, which is our case, the deposited energy density is higher at low velocities (Au : 0.09 MeV/amu) than at hight velocities (S: 2.34 MeV/amu). The ratios of the square ions velocities for both ions, which is proportional to the ratio of the cylindrical areas induced by each ions are the inverted ratio of the corresponding deposited energy density. Results that the energy density deposited by the gold ion is almost 700 times higher than the sulphur ion. It should be noted that the cell response to a given biophysical cue vary depending on anatomic origin of the vascular endothelial cells.

Moreover, the cells were cultured on the surface of artificial plastic and solution of nutrients. These conditions are not always alike to those in vivo, therefore it is very complicated to investigate the natural features and functions of cells. At certain ion fluences and 7 days of culturing times the cells formed a layer which almost covered the available surface. We determined a cell density 3.7 x 10^5 cells/cm^{-2} for the irradiation with sulphur beam compared with the density of 2.9 x 10^5 cells/cm^{-2} for the gold beam. Then the closed contact between this particular type of cells which only grow in a single layer, inhibited the proliferation process.

As a big advantage, cells seeded in biodegradable PLLA scaffold allow the harvest of intact single cell sheets. It is very important to keep cell-cell and cell-extracellular matrix connections because they are crucial for proper tissue developments. This is in contrast to some methods of cell adhesion disruption that usually include treatment with digestive enzymes, such as pancreatic trypsine, which destroy some of these important connections.

Concluding, this work compares the results of two beams with about the same deposited energy but different energy density and analyzes the ion fluence parameter that induces a total coverage of the surface. We found that the proliferation reaches a maximum for the sample irradiated with Au beam and with fluence more than 4 x 10^{10}. In the analyzed ion fluence range of sulphur beam, between 1 x 10^{10}. and 10 x 10^{10}, an almost constant value of proliferation, were measured. These results indicated that for Au irradiation there are some values of ion fluence or in other words a particular spacial distributions of ions spots that favor the adhesion and proliferation of cells [23]. In the case of the sulphur beam we founded a 20 % increase of cell density and a general better cell morphology compared with the gold irradiations. This observation suggests that the lower energy density which is deposited by the sulphur beam could produce a better sample surface for cell proliferation. Based on these results, it can be concluded that irradiated biodegradable polymers will become

promising materials for cell culture in which the cell adhesion, proliferation and morphology can be regulated by irradiation conditions. Due to its practical relevance, this study deserves an extensive and careful analysis in future investigation.

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