Nuclear Instruments and Methods in Physics Research B 365 (2015) 587–591

Contents lists available at ScienceDirect

Nuclear Instruments and Methods in Physics Research B

journal homepage: www.elsevier.com/locate/nimb

Studies of endothelial monolayer formation on irradiated poly-L-lactide acid with ions of different stopping power and velocity

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ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 31 August 2015
Accepted 7 September 2015
Available online 24 September 2015

Keywords:
PLLA
Irradiated polymers
Swift heavy ions
Cell adhesion
Cell proliferation

ABSTRACT

In this work we study cell viability, proliferation and morphology of bovine aortic endothelial cells (BAEC) cultured on poly-L-lactide acid (PLLA) modified by heavy ion irradiation. In a previous study comparing ions beams with the same stopping power we observed an increase in cell density and a better cell morphology at higher ion velocities. In the present work we continued this study using heavy ions beam with different stopping power and ion velocities. To this end thin films of 50 μm thickness were irradiated with 2 MeV/u and 0.10 MeV/u ion beams provided the Tandar (Buenos Aires, Argentina) and Tandetron (Porto Alegre, Brazil) accelerators, respectively. The results suggest that a more dense and elongated cell shapes, similar to the BAEC cells on the internal surface of bovine aorta, was obtained for stopping power of 18.2–22.1 MeV cm^2 mg^{-1} and ion velocity of 2 MeV/u. On the other hand, for low ion velocity 0.10 MeV/u the cells present a more globular shapes.

1. Introduction

Cell sheet engineering has arisen as an attractive approach to tissue engineering. In this approach, the formation of an endothelial cell monolayer and the confluent cell cultures are harvested from a variety of substrates as intact, tissue-like sheets consisting of the cells and their associated extracellular matrix [1]. The control of the substrate surface properties is a substantial step in the development and improvement of biomaterials for clinical applications. Interaction of the surface with the biochemical or biological environments is crucial for 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 [2]. Cells ability to anchor on a particular medium depends on the characteristics of the substrate as well as the culture conditions, cell type used and culture medium composition [3,2].

Among others, the most common substrate which is non-cytotoxic and biodegradable is polystyrene, commonly used to culture plates and three dimensional matrices. For preclinical studies in situ in experimental animals or in vitro in cell cultures, non resorbable synthetic polymers (polystyrene, polypropylene, polyethylene) are used [4,5]. Furthermore, many aliphatic types of polyesters have been extensively used for biomedical applications [6–9].

In particular, poly-L-lactide acid (PLLA) is a biodegradable polymer of great importance used in bioabsorbable sutures, drug delivery systems, biodegradable scaffolds and tissue-engineered blood vessels [10,11]. As the slow degradation of PLLA occurs by non enzymatic hydrolysis, it produces as end products, lactic acid and glycolic acid which are subsequently metabolized in the human body [12]. Nevertheless, there are drawbacks that result from their low bioaffinity. The difference in physico-chemical properties between hydrophobic polyesters and hydrophilic bio-active signals has a deep consequence on biomedical applications [13].

In order to achieve a higher attached cell density and to promote cell culture on biomedical materials, many studies have been reported to modify these biodegradable polymers using various approaches [14–18]. In particular, ion irradiation at different energies is an effective method used to provide specific surface properties in different polymer materials [19] and, in recent years, in PLLA [20,21]. The ion beam irradiation method modifies the surface characteristic and induces a more hydrophilic surface, without changing the degradation properties of PLLA [22]. In our previous

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http://dx.doi.org/10.1016/j.nimb.2015.09.021
0168-583X/

work [23] we studied the cell adhesion and proliferation as a function of ion beam fluence and the result was that a better monolayer formation was obtained with fluence larger than 4×10^{10} ions/cm^2.

The purpose of the present work is to explore the effects on cell adhesion and proliferation with other physical parameters such as: stopping power and ion velocity. To this end we irradiated foils of PLLA with a constant ion fluence of 7×10^{10} ions/cm^2 and used different ion beams with stopping power from 5 up to 22.1 MeV cm^2 mg^{-1} and with ion velocities of 2 and about of 0.10 MeV/u. The ion velocity determined the size of the ion spot and then induced different energy densities on the irradiated surface. In this work we study the endothelial cell proliferation, morphology and monolayer formation on irradiated PLLA with heavy ion beams during 1, 3 and 7 days of cell culture.

2. Experimental procedure

2.1. Polymer irradiation

Commercial grade films of PLLA polymer with a thickness of 50 μm manufactured by Goodfellow (England) were used "as received". The samples were irradiated with ion beams perpendicular to the surfaces in a vacuum of 10^6–10^7 Pa. The irradiation times for each sample varied as a function of the current intensity and the ion beam fluence was 7×10^{10} ions/cm^2. The irradiations were performed in the Tandar accelerator, Buenos Aires, Argentina (2 MeV/u) and the Tandetron accelerator, Porto Alegre, Brazil (0.10 MeV/u). Table 1 lists the ions, stopping power, velocity and energies used in the different experiments. To minimize sample heating during irradiation the current density was kept between 0.25 and 1.50 nA/cm^2. After irradiation, the samples were stored in a nitrogen atmosphere.

2.2. Cell culture and conditions

The adhesion and proliferation of BAEC endothelial cell line derived from the tunica intima of bovine aorta were studied in vitro, following the same procedure described in our previous work [23]. Cells were routinely cultured using the Polystyrene (TCPS P100) with a solution of Dulbecco Modified Eagles Medium (DMEM GIBCO) high glucose supplemented with Fetal Bovine Serum and antibiotic.

Then the cells were incubated at 37 °C with 5% of CO_2/95% of air and relative humidity of 95%. After a treatment with trypsine, the cells were harvested. Both, irradiated and control pristine PLLA samples, were sterilized prior to inoculation of cells. The samples were placed on the bottom of 24 wells TCPS with a density of 10^4 cells/cm^2 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 non irradiated PLLA films and then incubated for 1, 3 and 7 days. After these periods of time, we monitored the viability and biochemical activity of cell populations following the MTT assay. This test allows to determine the mitochondrial functionality and therefore the viability of the adhered cells. These cell viability measurements are proportional to the number of alive cells attached to the surface. This method only senses cells attached and alive and it is preferred over others which only counts cells.

Each of the relative cell viability measurements was obtained from the ratio between the average intensity of three wells which a near non irradiated PLLA (pristine sample) for normalization purpose. These procedures tried to avoid any possible difference of non homogeneity on the batch recipient during the culture experiments. The final reported cell viability values resulted from the average of six independent measurements, involving a total of 18 cells and 6 pristine samples, and the errors were calculated as standard deviations.

Table 1
Stopping power, ion beams and ion beam velocity used to irradiate the PLLA samples.

dE/dx (MeV cm^2 mg^{-1})	Ion	Energy (MeV)	Velocity (MeV/u)	Ion	Energy (MeV)	Velocity (MeV/u)
5.0	^{12}C	24	2	^{16}O	0.8	0.05
7.8	^{16}O	32	2	^{16}O	1.7	0.10
18.2	^{28}Si	56	2	^{63}Cu	7.5	0.12
22.1	^{32}S	64	2	^{63}Cu	9.5	0.15

2.4. Morphology and spreading

Morphology of endothelial cells cultured on the tested surface was then evaluated on micro photographs taken with an Olympus BX51 microscope. More experimental details are described in our previous work [23].

3. Results

The purpose of this work was to measure the number of cells attached to the irradiated surface as a function of different physical parameters such as: stopping power and ion velocity keeping the ion fluence constant at 7×10^{10} ions/cm^2.

Fig. 1 shows the cell viability values normalized to the pristine sample (100%) for three culture times (1, 3 and 7 days) as a function of the stopping power and for two ion beam velocities. Fig. 1 (a) shows the viability values for the ion velocity of 2 MeV/u; as it can be seen, in the first day of culturing time, the cells adhesion is less than 200% independent of the stopping power. In particular for the 5 and 7.8 MeV cm^2 mg^{-1} stopping powers the cell viability values fluctuated close to the pristine ones (100%). On the other hand, for the 18.2 and 22.1 MeV cm^2 mg^{-1} stopping powers, a sudden increase is shown in the number of cells which almost doubled their number at each culture time. Fig. 1(b) shows the relative cell viability for the average ion velocity of 0.10 MeV/u. For the first day of culture time, the result of an adhesion intensity of 250% up to 310% is nearly independent of the stopping power. For the 3 and 7 days of culture times the cells proliferated up to a value of 500%, except for 5.0 MeV cm^2 mg^{-1} stopping power, when they double the initial number of cells reaching 600%.

In addition to the cell viability intensity a relevant parameter to evaluate is the morphology of cells on a surface. To this end Fig. 2 shows the pictures of the BAEC cells over different substrates that reach, after 7 days of culture time the largest cell viabilities values of 600%. Fig. 2(b and e) compare the 1 and 7 days of culture time for 22.1 MeV cm^2 mg^{-1} stopping power and 2 MeV/u velocity and Fig. 2(c and f) for 5 MeV cm^2 mg^{-1} stopping power and 0.10 MeV/u velocity.

In Fig. 2(b and c) it can be appreciated the difference in cell densities, between the 2 and 0.10 MeV/u velocity, after 1 day of seeding. This difference is a factor of about three (see Fig. 1). In Fig. 2(e and f) the cells after 7 days of proliferation can be seen. The compactness of the cells indicate that there are so many cells that they are about to cover the total available surface. When the cells cover the total surface they develop a monolayer and the closed contact between this particular type of cells inhibit the proliferation process.

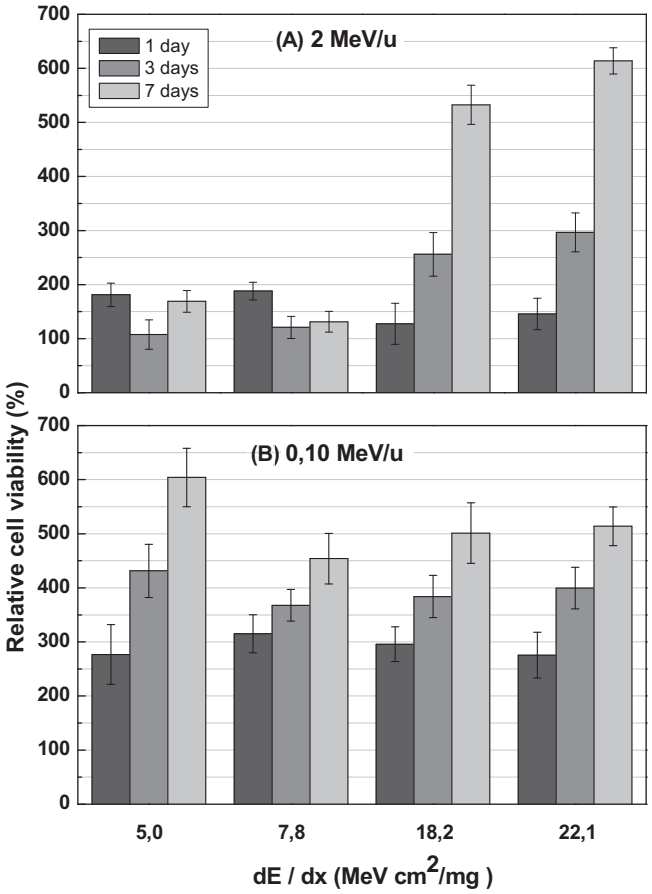

Fig. 1. The relative cell viability with respect to the pristine sample normalized as 100%. The proliferation of BAEC cell culture on PLLA for 1, 3 and 7 days as a function of stopping power. For constant ion fluence of 7×10^{10} ions/cm^2 and different ion beam velocity (a) 2 MeV/u and (b) 0.10 MeV/u.

Another comment about Fig. 2 is with respect to the cells shape or, in general, their morphology. As it can be seen from the pictures and the magnified image in the corresponding insets, Fig. 2(b and e) shows cells more closely packed and elongated, similar to an isolated BAEC cell or as endothelial cells in the bovine aorta, compared to the cells shown in Fig. 2(c and f) in which a more globular shape aspect predominates.

Concluding, Fig. 2(a and d) shows a typical picture of the pristine sample used for normalization purposes (100%) for 1 and 7 days of culture time, respectively. As it can be observed it shows same groups of tightly adhered cells surrounded by an empty surface. This is clearly different to the cells attached on an irradiated sample after also 1 day Fig. 2(b and c) and 7 days Fig. 2(e and f) of seeding.

4. Discussion and conclusion

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 work we analyzed, in addition to cell adhesion, its evolution as a function of time. We measured the cell proliferation parameter and observed their morphology until it covered the total irradiated surface.

The ion beam deposits energy in concentrated surface spots, randomly distributed. The dissipation of the ion energy is mainly mediated by energetic electrons (delta rays) and 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 [24] hence for the same stopping power; the deposited energy density is higher at low velocity (0.10 MeV/u) than at high velocity (2 MeV/u). These different deposited energy densities induced different morphological and chemical changes at the ion spots randomly distributed over the polymer surface.

In a previous work, we studied [22] the chemical change induced by swift heavy ions using different techniques and measured that the contact angle was reduced making a more hydrophilic surface. Then these active irradiated surface were seeded with cells on a culture medium with serum solution. It is well known that cell adhesion is mediated by the formation of an adsorbed protein layer, coming from the complex serum medium, with different proteins such as: fibronectin, albumin, collagen, etc. [25,26]. These produced the anchoring place which the cells evaluate before starting the process of surface adhesion [27,28]. At the beginning, the cell develops specific places called focal adhesion, which are the anchoring points to the surface. After the adhesion takes place, it starts to sense the surroundings using active extension of the membranes beyond the cell edges (filopodia) into unoccupied areas. If the neighborhood is appropriate, the cell starts the proliferation process [27,29].

The purpose of this work is to analyze the culture cells on PLLA surfaces with different stopping power and ion beam velocities and constant spatial distributions of ion spots (fluence) by measuring the proliferation and morphology of the cells.

As it can be seen in Fig. 1 and for the first day after seeding, the cell viability values fluctuated around: less than 200% (Fig. 1(a), 2 MeV/u) and 300% (Fig. 1(b), 0.10 MeV/u) almost independently of the stopping power values. These results suggest that the difference on the initial cell adhesion can be correlated to the difference on the ion velocity parameter.

On the other hand, observing the proliferation parameter Fig. 1 (b) the cell viability reaches the value of 450% up to 600% after 7 days of culturing time independent of the stopping power. Instead, the Fig. 1(a) shows proliferation only at stopping power 18.2 and 22.1 MeV cm^2 mg^{-1}.

The morphology analysis shows cells more closely packed and elongated for 2 MeV/u velocity and with similar shapes to the BAEC endothelial cells on the internal surface of bovine aorta under hemodynamic forces [30]. On the other hand for low velocities 0.10 MeV/u, the cells present a more globular shape.

From these previous results we can suggest that there are certain range of parameters that favored a development of a monolayer of BAEC cells on the PLLA surface. These are, stopping power between 18.2 and 22.1 MeV cm^2 mg^{-1} and ion velocity of 2 MeV/u.

A possible explanation for these effect can be attributed to the different ion spot radius or deposited energy density. The ion spot of 2 MeV/u has a radius greater than the low ion velocity 0.1 MeV/u. The larger zone, with a much lower deposited energy density, induced an extended hydrophilic zone with active chemical species [31]. This favored the attachment of proteins or other chemical species, coming from the culture medium, that mediated the highly complex cell adhesion process.

Let us discuss these last results suggesting ideas for future research: the large initial cell adhesion, for low ion velocity (0.10 MeV/u), covers most of the surface leaving little space to newly divided cells. Fig. 1(b) shows than after 7 days of culturing times they can only double their number. Instead, for high velocity irradiation (2 MeV/u) the cells proliferated increasing their number four to five times until they cover the surface. This suggests that the large initial adhesion could leave the cells tightly grouped not inducing cell division, a complex process described by A. de Beer et al. and references therein [32].

Fig. 1(a) shows clearly the effect of the stopping power, the cell proliferates only for the values 18.2–22.1 MeV cm^2 mg^{-1}. This indicates that this particular deposited energy induces the necessary

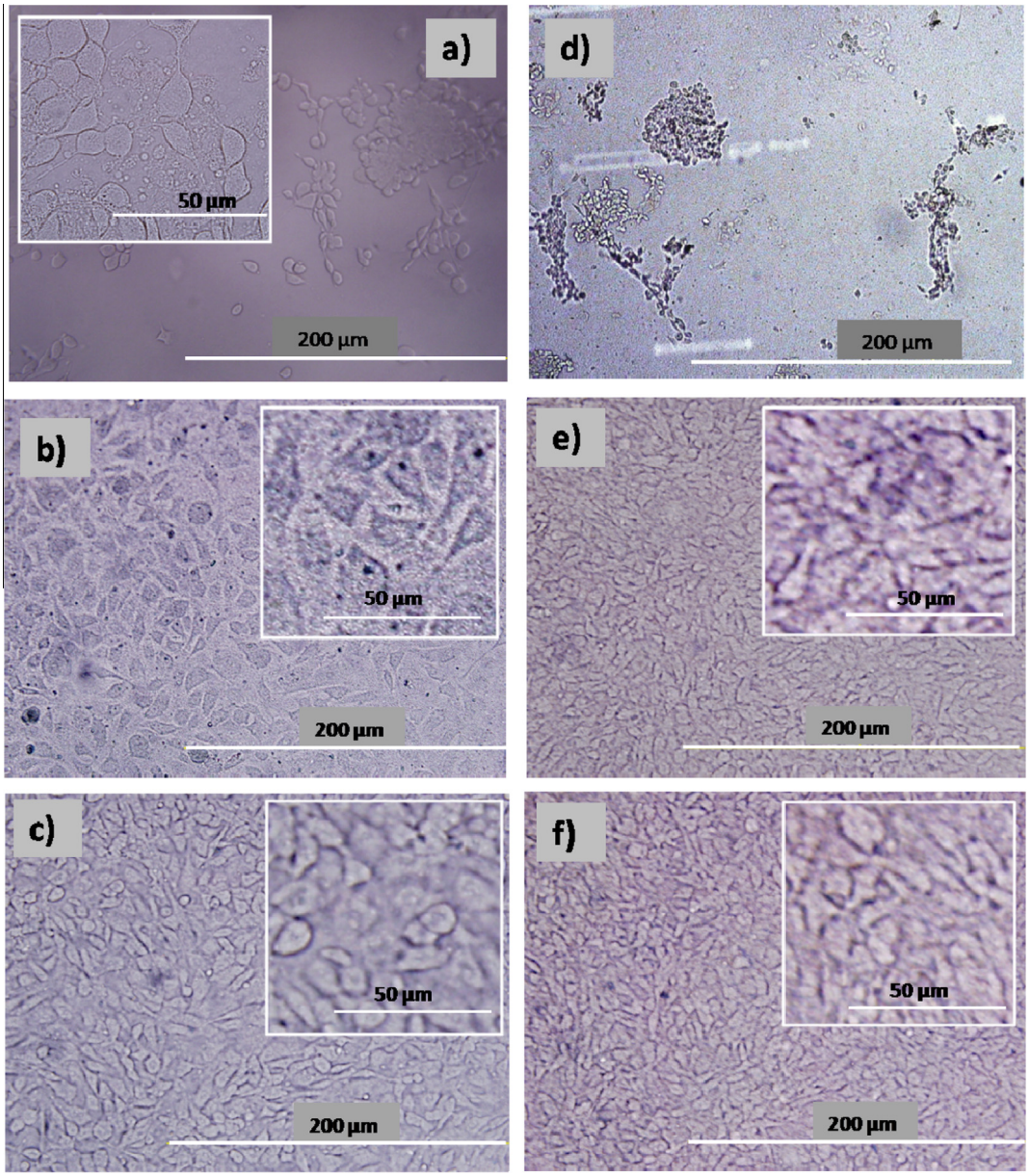

Fig. 2. Phase contrast micro photographs of endothelial cells attached on PLLA of the pristine sample after 1 day (a) and 7 days (d) of seeding used for normalization purpose (100%). As it can be observed shows same groups of tightly adhered cells surrounded by an empty surface. Clearly different to the cell attached on irradiated sample after also 1 day of seeding (b and c) and 7 days (e and f) of culture time and for different irradiation conditions: (b and e) for 22.1 MeV cm^2 mg^{-1} stopping power and 2 MeV/u velocity and (c and f) for 5 MeV cm^2 mg^{-1} stopping power and 0.10 MeV/u velocity. The inset shows a four times amplified view (50 µm) of the photographs. See text for details.

conditions for the cell to start the adhesion process. To explore further we need to measure, using different experimental techniques, the chemical composition difference on the ion spot after the irradiation and also after it is in contact with the serum. The adhesion process is a very complex process which depends on the substrate as well as the culture conditions, cell type used and culture medium composition.

Concluding, the ion irradiation constitutes an alternative way to modify the polymer surface. It is rather simple and fast and it can be improved with additional chemical treatments of the irradiated surface [26,33,34].

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