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Evaluation of cell behavior on modified polypropylene with swift heavy ion irradiation

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ABSTRACT

Ion beam irradiation is a well known means to change the physico-chemical properties of polymers, and induced bio and citocompatibility in controlled conditions and in selected areas of surface. However, the enhancement of cell adhesion on a modified substrate does not mean that the surface is adequate for functional cells. The purpose of the present work is to study proliferation, changes in cytoskeleton and cell morphology on substrates as a function of irradiation parameters. We irradiated polypropylene with sulfur (S) ion-beam at energies of 110 MeV with fluences between 1×10^6 and 2×10^{10} ions cm^{-2}. NIH 3T3 cells were cultured on each sample. Cell morphology was observed using phase contrast microscopy and cytoskeleton proteins with fluorescence microscopy. The analysis show different cellular responses as a functions of irradiation parameter, strongly suggests that different underlying substratum can result in distinct types of cytoskeleton reorganization.

1. Introduction

Synthetic polymers have been often applied in different fields for their excellent bulk properties and, within this category; biomaterials are widely used in tissue culture technologies and medicine. Biomaterial surface chemistry and nanoscale topography are important for many potential applications like substrates for cell culture due to their strong influence on cell behavior. Numerous in vitro experiments have shown that the cell functions such as adhesion, cell migration, proliferation and gene expression depend on the physicochemical properties of biomaterial surfaces [1–3]. Hence, in many cases an additional modification of polymer surfaces is required to achieve desired surface properties such as better cell adhesion and increased bioactivity; while maintaining the polymer bulk characteristics [4,5].

Ion beam irradiation is an effective surface modification technique, which uses heavy ions to alter the physicochemical properties of the surface of polymers inducing new chemical functional groups as well as changes in surface free energy and topography [6,7]. In particular, the irradiation of nonpolar polymers such as polypropylene (PP), lead to modified material surface that improve the interaction with macromolecules which act as a mediators of cell adhesion on a substrate. Due to its hydrocarbon constitution and its tolerance by the human body, PP is widely used as non absorbable surgical suture and surgical repair of abdominal parietal defects as prostheses. However, one great limits of PP is the lack compatibility for the cells. Its chemical inertness results in hydrophobic surface and limits its potential applications like substrates for cell culture; so it is necessary to introduce some reactive groups on the polymer surface. Our previous study reported that changes produced by ion beam irradiation into a PP polymer have an evident effect on the initial adhesion of Mouse embryonic fibroblasts NIH 3T3 [8]. Cell adhesion to extracellular matrix (ECM) and support substrates has functional implications. The adhesion process in the biomaterial involves short term events like physicochemical linkages between cells and materials through ionic forces, van der Waals forces, etc. On the other hand the adhesion phase, which involves various biological molecules: ECM proteins, cell membrane receptors (like integrins) and cytoskeleton proteins and taken a large period of time. The sites of adhesion between cultured cells and substrate are called focal contacts [9]. Adhesion reactions are very important cellular functions, as it is known, integrins located within the adhesions and actin cytoskeleton linked to integrins, are involved in signal transduction pathways that regulate cell proliferation and survival and promote the action of transcription factors and consequently regulating gene expression [10]. It is know that

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in absence of proper cell adhesion, cells undergo programmed cell death or apoptosis [11]. Slender cytoplasmic projections composed of actin filaments called filopodia, which extend from the leading edge of migrating cells and form focal contact with the substrate, linking it to cell surface. When a suitable site for adhesion has been detected, focal adhesion and mature actin fibers are formed, it implies changes of the cellular cytoskeleton which was reorganized stabilizing the contact. In a like manner, cell migration requires the coordinated changes in the actin cytoskeleton and the controlled formation of cell substrate adhesion sites. Cells with a low motility form strong focal adhesions while motile cells form less adhesive structures [12]. The ability of the substrate to promote the formation of focal contacts and the development of the cell cytoskeleton are important for the performance of the biomaterial because is closely related to cell behavior to their surface. Although there have been numerous studies on modification of polymers by irradiation for cell attachment, few have described the morphology and conformation of cytoskeleton proteins of the cell adherers. In the present study, we have examined the effect of irradiated surface on cell attachment, proliferation, spread out and cytoskeleton organization of NIH 3T3 fibroblasts. The different responses are evaluated in terms of the deposited energy in concentrated surface spots by heavy ion in a polymer at different irradiation conditions.

2. Experimental procedure

2.1. Substrate irradiation

The polymer sample used in the present study is of commercial grade manufactured by Goodfellow (England): PP biaxially oriented with a thickness of 8 μm. Polymer films were irradiated in a vacuum of 10^{-6} to 10^{-7} Pa with ion beams perpendicular to the surfaces. A heavy ions beam (S, energy 110 MeV) was provided by the Tandar accelerator – CAC, Buenos Aires, Argentina. The treatment times periods at each samples were varied, as a function of the current intensity and required ion fluences of 1×10^6, 2×10^9, 5×10^9, 1×10^{10} and 2×10^{10} ions cm^{-2}. The ion fluences were measured in situ with a Faraday cup. The ionic current density was between 15 and 40 nA, to minimize sample heating. After irradiation the samples were stored in air.

2.2. Cell culture and conditions

Fibroblasts NIH 3T3 cell line were maintained routinely in Dulbeccos Modified Eagles Medium (DMEM GIBCO) supplemented with 10% FBS (Fetal Serum Bovine, Natocor, Argentina) and 1% antibiotic solution. Cells were incubated under standard cell culture conditions. Both ions irradiated and control pristine polypropylene were sterilized in 70% C_2H_5OH and rinsed with de-ionized water and phosphate buffered saline (PBS). Then, they were placed on the bottom of a 24 wells TCPS, covered with a sterilized Viton ring to prevent floating. The cells were harvested using trypsine 0.25% (Sigma)–0.02% EDTA in PBS. After that, fibroblasts were seeded onto 24-wells TCPS covered with the studied polymers, with a density of 1×10^4 cells cm^{-2}. The culture wells were incubated under the same conditions above.

2.3. Cell adhesion and proliferation assays

In order to examine the effects of irradiation treatment on the cellular behaviors of NIH 3T3, the cells were seeded onto irradiated and unirradiated PP films and incubated for 24, 48 and 72 h for attachment and proliferation assay. After those periods of time, cell viability and proliferation on each specimen was measured by MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product). The optical density of the formazan solution thus obtained in each well was measured in a plate reader Beckman Coulter DTX 880 at 560 nm; it is related to adhered cells. All cells assays carried out in triplicate.

2.4. Morphology and cytoskeletal evaluation

Morphology of NIH 3T3 fibroblasts cultured on the tested surface was then evaluated on microphotographs taken under an Olympus BX51 microscope (objective 100× and 10×). Components of the cytoskeleton can be imaged using fluorescent staining techniques. To visualize actin filament structure, attached cells were fixed using paraformaldehyde (4%) for 15 min, washed with PBS, permeabilized with 0.5% Triton X-100 for 15 min and block nonspecific binding using 3% non-fat dry milk in PBS. Then, fixed cells were stained with rhodamine phalloidin according to manufacturers directions. The nucleus was stained with DAPI, 4,6-diamino-2-phenylindole (Molecular Probes). The specimens were examined using an Olympus BX51 microscope.

3. Results and discussion

Proliferation and viability of NIH 3T3 cells on various PP substrates were determined by MTT tetrazolium assay after cultured for 24, 48 and 72 h. Fig. 1 displays the results of fibroblasts adhesion on PP modified with different ion fluence. The values obtained were measured as a percentage of those from control cultures on the unmodified polymer. The proliferation index at 48 and 72 h was defined as the ratio of absorbance measurement at 48 h and 72 h divided by the absorbance at 24 h and are showed in Fig. 2. On the irradiated polypropylene, initial attachment was significantly higher than that of unirradiated polymer and the highest was obtained at a maximum fluence examined (2×10^{10} cm^{-2}). In addition, for most of the fluences examined the optical density decreased at 48 and 72 h except at 2×10^9 cm^{-2}.

As can be seen in Fig. 2, cell adhesion increased at 2×10^9 cm^{-2} improving proliferation. The trend seen is consistent with the results shown in Fig. 1 because cell adhesion decreased with the time in a fluence-dependent manner to indicate cellular death and consequently decreased in proliferation. An important fact was that for the 2×10^9 cm^{-2} condition, where a low initial adhesion result in a better proliferation and as will be seen in an optimum morphology and spread out. These

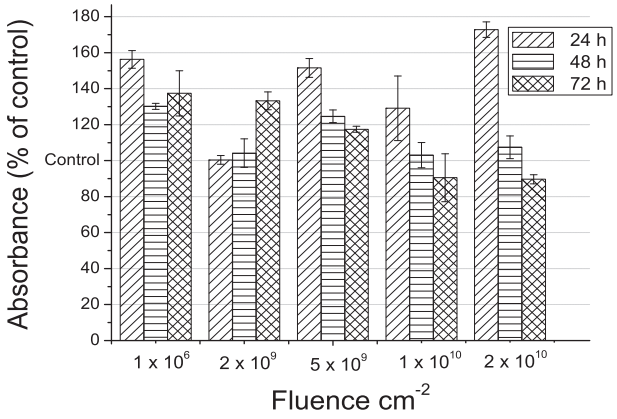

Fig. 1. The cell attachment trends of NIH 3T3 fibroblasts cultured on various PP supports by MTT-tetrazolium measurements. Absorbance values of cells from the ion modified polymers are expressed in % of values obtained from control cells grown on pristine PP. The data showed represented the average of three measurements.

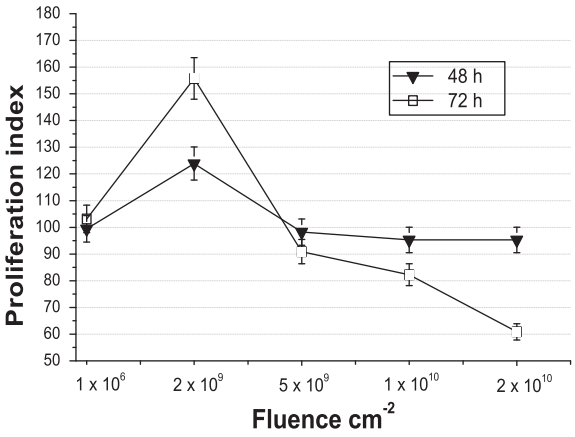

Fig. 2. Effects of ion beam treatment on proliferation of NIH 3T3 fibroblasts according to the fluence of irradiation. Results indicated that cell proliferation is better at 2 $\times 10^9$ and then decreased with the fluence.

results was in agreement with the know fact that the cell shape has an important influence on cell proliferation, and cell spreading correlates with higher rates of proliferation [13].

The overall cell morphology of fibroblasts adhering on the irradiated polymers obtained with 100× and 10× objectives is shown in Fig. 3, illustrating the grades considered to evaluate the behavior of the cells. The cell attachment, particularly the focal adhesion, is directly related to filopodia behavior. On irradiated treated surfaces at 1×10^6, 2×10^9, 5×10^9 cm^{-2} there are extensive protrusions on the border of the cells and the phenotype obtained was lengthened and fusiform (Fig. 3b and c); whereas on the high fluences irradiated surface there are clearly a very limited number of protrusions extruding from the cell edge (Fig. 3 a). The morphology observed on pristine PP sample is the same as the high fluences in which attachment cells retaining spherical morphology. In these cases, the stretch of the filopodia is insufficient and the cells have a tendency to formed multicellular aggregates. Cells responded better to the 2×10^9 irradiated surface: at 10× samples shows high grade of spreading on substrates and their phenotype manifest filopodia corresponding to normal fibroblasts morphology.

Filopodia protrusions have the functions of contacting the external environment, anchoring appendages that hold the cell bodies in place and the intercellular connectives that can join cell bodies. The process of cell attachment starts when the integrins receptors sense the environment or ECM surface and then send signals to the cell nucleus to indicate a surface for the cell to attach

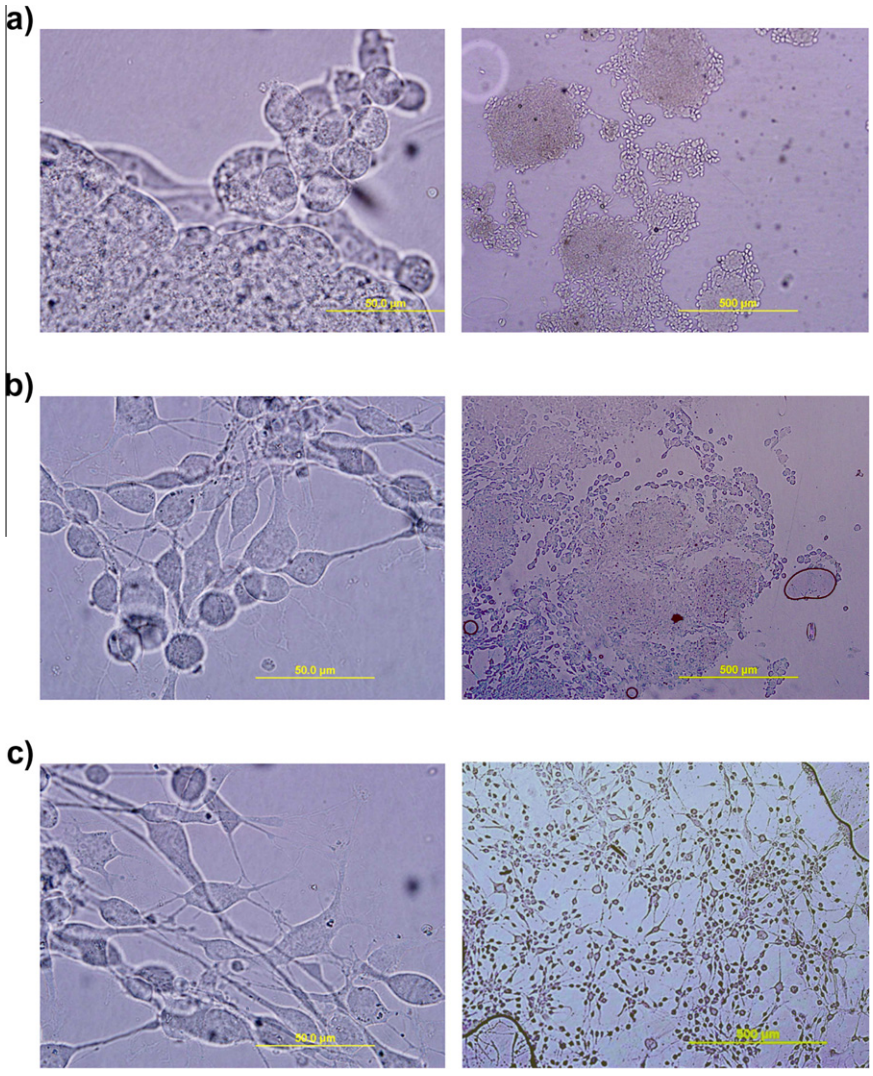

Fig. 3. Microscopic images at 100× (left lane) and 10× (right lane) of the fibroblasts attached on PP irradiated for different fluences. (a) 2×10^{10} cm^{-2}: cells were mostly rounded (b) 5×10^9 cm^{-2}: showed filopodial protrusions, though even formed conglomerated. (c) 2×10^9 cm^{-2}: cells exhibited a high grade of spreading on the substrate. See text for details

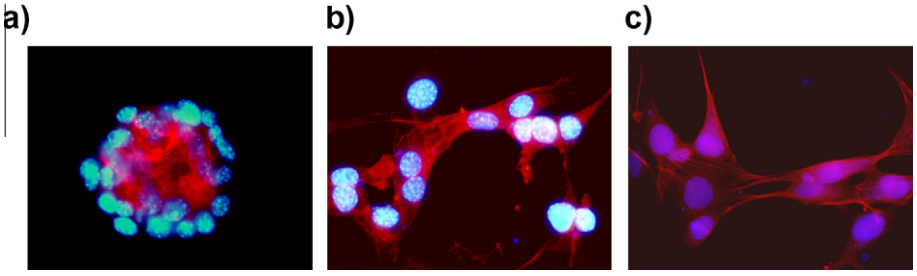

Fig. 4. Fluorescence micrographs of spread NIH 3T3 cells stained for F actin by rhodamine-conjugated phalloidin (red) and the nucleus with DAPI (blue) after 72 h incubation. Cytoskeletal organization was altered in spread cells on substrates corresponding to fluences from 5×10^9 ions cm^{-2} (a and b). At 2×10^9 cm^{-2} can be observed stress fibers formation in attachment cells (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[14]. Irradiated polymers has been well known to produce surface modifications by the no homogenous distribution of ions. The cell receptors sensed different environmental from the unirradiated surface than the treated surface, and finally formed focal contacts which differ according to the PP irradiation fluences. Compared with other types of radiation, such as electrons or gamma rays, which perturb the materials homogenously, the heavy ions instead deposited energy in concentrated surface spots, randomly distributed. The ion energy dissipation is mediated by energetic electrons and the dose distribution around the ion track has been the subject of several studies [15,16]. From these works it is possible to obtain the area where about 80% of the total available energy is deposited. In particular for a ion energy of 3.4 MeV/amu result a radius of about 0.1 μ [17]. If we assumed that most of the chemical change occur in this area then the number of ions necessary to cover the unit area, without overlap, result 3×10^9. Due to the random characteristic of the ion hits (Poisson statistic) at this particular fluence exist overlap and then the irradiated unit area is divided in different zones such as: pristine (45%), single hit (25%) and overlap of two or more hits (30%) areas [18,19]. The proliferation measurement shows a peak at about the some ion fluence discussed previously (2×10^9 cm^{-2}, Fig. 2) suggesting that the distribution of single spots and pristine areas favor the spreading of cells on the irradiated surface.

In this study, we also observed changes in F-actin cytoskeleton using fluorescence microscopy. The cytoskeletal results also show a change response from organization of cytoskeleton for cell attachment on substrates irradiated as a function of fluences. At high fluences, actin was generally diffuse, overall cytoskeletal structure were much less pronounced showed a great alteration compared with the stress fibers observed in cells cultured on PP 2×10^9 cm^{-2}. Representative examples of cell stained for F actin are show in Fig. 4. Conformation of the actin cytoskeleton is essential to the maintenance of cell shape and cell adhesion: it is known that changes in cell shape are mediated by alterations in the cytoskeleton.

4. Conclusion

Most of the previous studies measured only the growth of cells. Instead our work focus in addition, on the cells reactions on irradiated surface. Cell shape show large differences with irradiations conditions strongly suggests that different underlying substrates can result in distinct types of cytoskeleton reorganization, exercise influence on cellular responses.The cell evolution is related to filopodia behavior and changes in cell shape influences proliferation. Due to the no homogenous distribution of ions hits, the cellular receptors may selectively sensed ion hits with varying degrees of overlap. Then leads to the formation of focal contacts which differ according to the irradiation conditions. Cell adhesion is increased to 2×10^9 cm^{-2} which improve the proliferation while at higher fluences adhesion decreases over time, which indicate cell death (necrosis/apoptosis) with the consequent decrease in proliferation. The changes of cell behaviors, at certain fluences, could be understand because the cells found a better surface to attach and with more favorable landscape for cell filopodia and adhesion proteins to migrate. Concluding the present works study, to our knowledge for the first time, the proliferation parameter as a function of ion fluence and show the existence of a maximum or optimum value. The mechanism underlying this phenomena is not clear and certainly deserves further research.

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References

[1] L.T. Allen, E.J. Fox, I. Blute, Z.D. Kelly, Y. Rochev, A.K. Keeanan, K.A. Dawson, W.M. Gallagher, Proc. Natl. Acad. Sci. USA 100 (2003) 6331–6336.
[2] X. Liu, J.Y. Lim, H.J. Donahue, R. Dhurjati, A.M. Mastro, E.A. Vogler, Biomaterials 31 (2007) 4535–4550.
[3] J.M. Anderson, Annu. Rev. Mater. Res. 31 (2001) 81–110.
[4] J.M. Grace, L.J. Gerenser, J. Dispers. Sci. Technol. 24 (2003) 305.
[5] J.H. Lee, J.W. Lee, G. Khang, Lee H.B. Jung, Biomaterials 18 (1997) 351–358.
[6] K. Rockov-Hlavckov, V. Svorck, L. Backov, B. Dvornkov, J. Heitz, V. Hnatowicz, Nucl. Instrum. Meth. Phys. Res. B 225 (2004) 275–282.
[7] L. Backov, V. Mare, M-G. Bottone, C. Pellicciari, V. Lis, V.J. Svorck, Biomed. Mater. Res. 49 (2000) 369–379.
[8] C.R. Arbeitman, M.F. del Grosso, I. Ibañez, G. García Bermúdez, H. Durán, V.C. Chappa, R. Mazzei, M. Behar, Nucl. Instrum. Meth. Phys. Res. B 268 (2010) 3059–3062.
[9] K. Burridge, M. Chrzanowska-Wodnicka, Annu. Rev. Cell Dev. Biol. 12 (1996) 463–519.
[10] A.K. Howe, A.E. Aplin, R.L. Juliano, Curr. Opin. Genet. Dev. 12 (2002) 30–35.
[11] J.E. Meredith Jr, M. Schwartz, Trends Cell Biol. 7 (1997) 146–150.
[12] A. Huttenlocher, R.R. Sandborg, A.F. Horwitz, Curr. Opin. Cell Biol. 7 (1995) 697–706.
[13] C.S. Chen, M. Mrksich, S. Huang, G.M. Whitesides, D.E. Ingber, Science 276 (1997) 1425–1428.
[14] P.K. Mattila, P. Lappalainen, Nat. Rev. Mol. Cell Biol. 6 (2008) 446.
[15] T.A. Tombrello, Tucl. Instrum. Meth. Phys. Res. B 94 (1994) 424.
[16] M.P.R. Waligorski, R.N. Hamm, R. Katz, Tracks Nucl. Radiat. Meas. 11 (1986) 309.
[17] R. Spohr, Ion Tracks and Microtechnology: Principles and Applications, Ed. Viewed (1990).
[18] H. Kudoh, M. Celina, G.M. Malone, R.J. Kaye, K.T. Gillen, R.L. Clough, Radiat. Phys. Chem. 48 (1996) 555.
[19] C. Riedel, R. Spohr, Radiat. Eff. 42 (1979) 69.