Adhesion and morphology of fibroblastic cells cultured on different polymeric biomaterials

C. B. LOMBELLO[1], A. R. SANTOS JR.[1], S. M. MALMONGE[2], S. H. BARBANTI[3], M. L. F. WADA[1], E. A. R. DUEK[4*]
[1]*Department of Cell Biology, Institute of Biology, Campinas, SP, Brazil*
[2]*Labiomec, DDPP/Center of Technology, Campinas, SP, Brazil*
[3]*Department of Materials Engineering, Faculty of Mechanical Engineering, State University of Campinas (UNICAMP), Campinas, SP, Brazil*
[4]*Department of Physiological Sciences, College of Biological Sciences, Pontificial Catholic University of São Paulo (PUC-SP), Sorocaba, SP, Brazil*
E-mail: eliduek@fem.unicamp.br

Cell adhesion is influenced by the physical and chemical characteristics of the materials used as substrate for cell culturing. In this work, we evaluated the influence of the morphological and chemical characteristics of different polymeric substrates on the adhesion and morphology of fibroblastic cells. Cell growth on poly (L-lactic acid) [PLLA] membranes and poly(2-hydroxy ethyl methacrylate) [polyHEMA], poly(2-hydroxy ethyl methacrylate)-cellulose acetate [polyHEMA-CA] and poly(2-hydroxy ethyl methacrylate)-poly(methyl methacrylate-co-acrylic acid) [polyHEMA-poly(MMA-co-AA)] hydrogels of different densities and pore diameters was examined. Cells adhered preferentially to more negativaly charged substrates, with polyHEMA hydrogels being more adhesive than the other substractes. The pores present in PLLA membranes did not interfere with adhesion, but the cells showed a distinctive morphology on each membrane.

Introduction

Biomaterials play an important role in tissue engineering by serving as scaffolds to guide tissue regeneration by releasing medicines and growth factors to stimulate the tissue response, or to create a new functional structure when damaged tissue does not regenerate. Biomaterials can also be used in cell culture techniques to create tissue-like structures that simulate the mechanics and physiological characteristics of tissues *in vivo*.

Materials used in implants can be classified as biostable or permanent and bioabsorbable or temporary [1]. Biostable materials include prostheses, which are used to substitute damaged body parts for an unspecified period of time [2]. The materials used in these cases need to retain their mechanical and chemical properties *in vivo* for years or decades [1]. In many cases, however, only the temporary presence of a biomaterial support is required during tissue replacement or to guide tissue growth during wound healing. Bioabsorbable polymers are components that are degraded *in vitro* and *in vivo*, and disappear with time as the tissue is being repaired [2]. In both cases, a knowledge of the pattern of cell adhesion, growth, and differentiation on these biomaterials is important.

Poly(L-lactic acid) (PLLA) is biodegradable polyester frequently used as a support in cell culture or for the experimental treatment of damaged tissues, mainly because of its good biocompatibility [3–6]. PLLA degradation occurs by hydrolyses, which results in a gradual release of the degradation monomers [7]. PLLA devices have been prepared by techniques such as phase inversion [8], addition of salt particles [9], addition of plasticizer[10], incorporation of CO_2 [11], and by solution casting [12].

Another biomaterial commonly used in tissue engineering is poly(2-hydroxy ethyl methacrylate) (polyHEMA). This compound forms a hydrogel consisting of a three-dimensional polymeric network that swells in water without dissolving [13]. PolyHEMA shows similarities with the extracellular matrix, and thus represents a good model for cell culture studies of tissue restoration. Indeed, polyHEMA copolymers that simulate the articular cartilage matrix have been tested as alternative permanent prostheses. Chemical modification have been used to evaluate the hydrophobic/hydrophilic properties of polyHEMA in order to establish which changes affect cell adhesion and growth [14–18]. The presence of a $-COO^-H^+$ group in the copolymer structure introduces a higher negative charge changes the cell–polymer interactions [19, 20].

In this work, we examined the influence of structural variations in PLLA and polyHEMA on the adhesion,

*Author to whom all correspondence should be addressed: Eliana Aparecida de Rezende Duek, Departamento de Engenharia de Materiais, Faculdade de Engenharia Mecânica (FEM), Universidade Estadual de Campinas (UNICAMP), Cx. Postal 6122, CEP13083-970, SP, Brazil.

growth and morphology of cultured fibroblastic cells. The materials tested included dense and porous PLLA membranes of known in pore diameters, or polyHEMA and its copolymers poly(2-hydroxy ethyl methacrylate)-cellulose acetate [polyHEMA-CA] and poly(2-hydroxy ethyl methacrylate)-poly(methyl methacrylate-co-acrylic acid) [polyHEMA-poly(MMA-co-AA)].

Materials and methods

Preparation of biomaterials

Dense PLLA membranes

Poly(L-lactic acid) [PLLA] membranes were prepared by dissolving PLLA (high purity for medical use, Mw = 300,000 Da, obtained of Medisorb Technologies International LP, Cincinnati, OH, USA) at room temperature in 10% chloroform solution (w/v) (from Merck KGaA, Darmstadt, Germany). The polymer solution was then transferred to Petri dishes which were dried by casting in a closed chamber connected to a flow of compressed dry air (0.01 nm^3/h) for 24 h.

Porous PLLA membranes

The porous PLLA membranes were prepared by dissolving PLLA in 10% chloroform solution (w/v) at room temperature. Forty grams of sodium citrate with grain diameter < 45 μm; 180–250 μm and 250–350 μm (Fluka Chemicals, Netherlands) were added in portions of 10 g to 70 mL of PLLA–Chloroform solution to produce a film with a pore volume of approximately 80%. The drying procedure was as described for the non-porous film. After drying, the films were washed in demineralized water for 24 h to remove the salt and subsequently washed for 6 h in ethanol. In both cases, the polymers were vacuum dried and maintained in a dessicator for 5 days to guarantee the total removal of solvent.

PolyHEMA hydrogels

Three hydrogels, namely, poly(2-hydroxy ethyl methacrylate) [polyHEMA], poly(2-hydroxy ethyl methacrylate)-cellulose acetate [polyHEMA-CA], and a poly(2-hydroxy ethyl methacrylate)-poly(methyl methacrylate-co-acrylic acid) [polyHEMA-poly(MMA-co-AA)] were used in the form semi-interpenetrating networks (sIPN). Hydrogel samples were obtained as 2 mm thick sheets by thermal polymerization. The monomer, crosslinking agent (1.0% w/w) and thermal initiator (0.5% w/w) were mixed by stirring and poured into a glass mold for polymerization. In the case of mixed sIPN, the linear polymer, cellulose acetate or poly(MMA-co-AA) was dissolved in the HEMA monomer to give a solution with a final polymer concentration of 5% (w/w). After synthesis, the hydrogels were washed in distilled/deionized water to remove residual monomer and initiator, and then washed in 0.15 M NaCl until a constant pH 7.0 was reached. The uptake of NaCl the different hydrogel samples was measured and expressed as the percentage of saline solution within the gel at equilibrium. The fixed negative charge density (mEq COO^- /gel gram) was measured by titration.

For use in cell culture, the biomaterials were sterilized and the sterility confirmed. The samples were washed then three times in Ham F-10 medium without fetal calf serum and incubated in culture medium for 24 h at 37 °C before cell addition.

Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*), were obtained from the Adolfo Lutz Institute, São Paulo, Brazil. The cells were cultured in Ham-F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, from Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37 °C. Vero cells are recommended for studies of cytotoxicity and cell-substratum interactions in biomaterial research [21, 22].

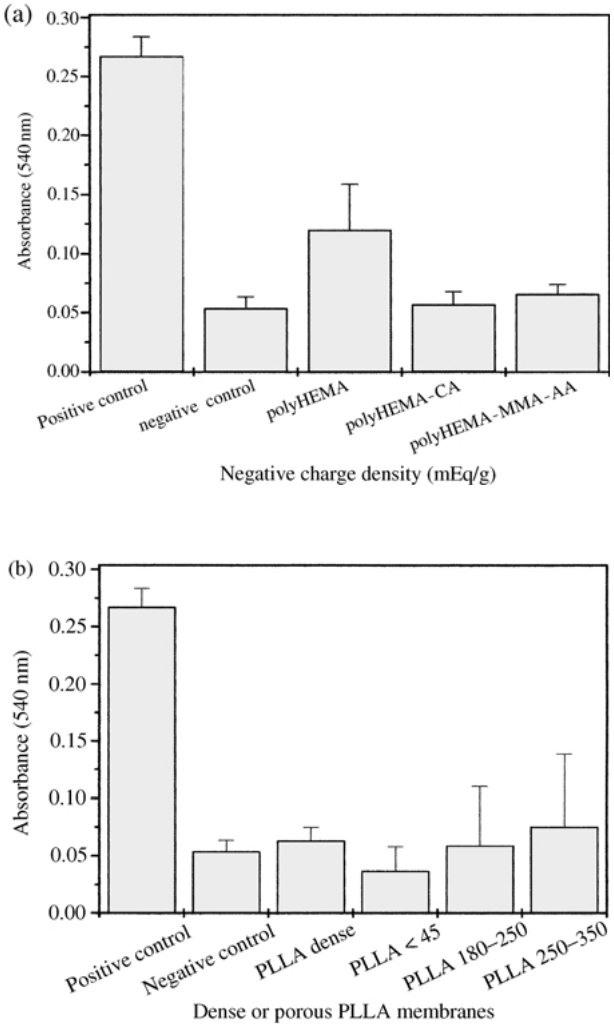

Figure 1 Cell adhesion to different polymeric biomaterials after 2 h incubation. (a) Cell adhesion on hydrogels of different negative charge densities: polyHEMA (0.017 mEq/g), polyHEMA-CA (0.02 mEq/g) and polyHEMA-(MMA-co-AA) (0.11 mEq/g). Polypropylene and silicone were used as a positive and negative controls, respectively, (b) cell adhesion on porous PLLA membranes of different diameter pores.

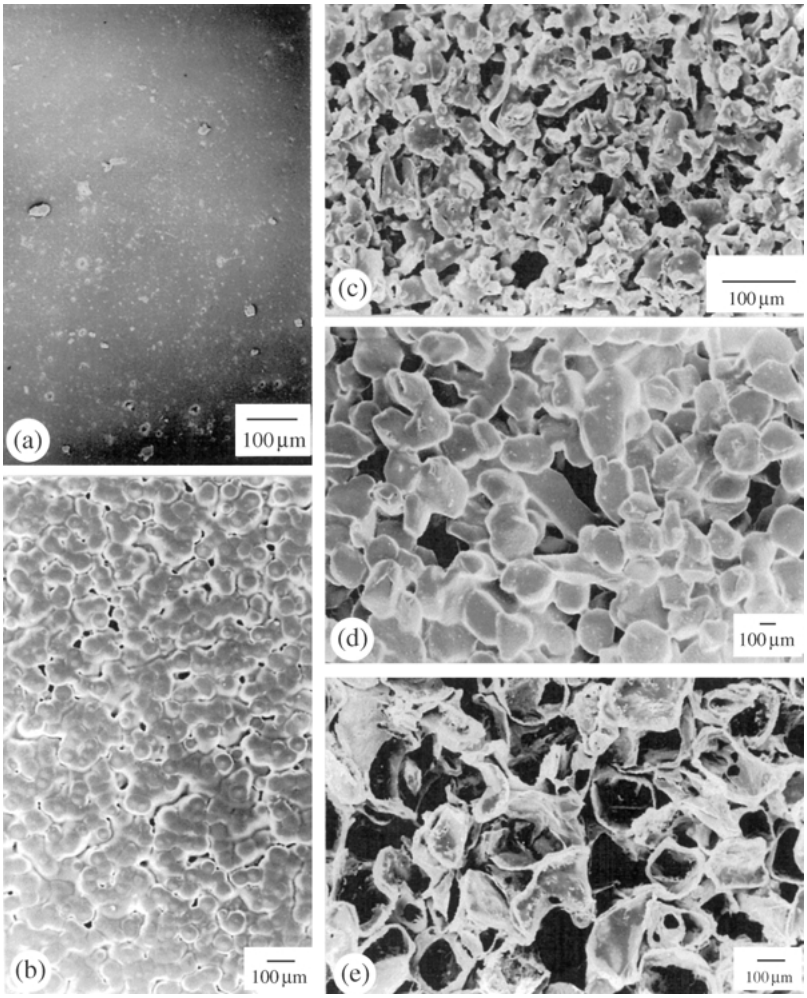

Figure 2 Scanning electron microscopy of different biomaterials used as substrate in cell culture. (a) PolyHEMA with a dense membrane surface, (b) dense PLLA membrane, (c) PLLA membrane with a pore diameter $< 45\,\mu m$, (d) PLLA membrane with 180–250 μm pores, (e) PLLA membrane with 250–350 μm pores. Barr: 100 μm.

Cell adhesion

A modification of Mosmann's method [23] was used. Briefly, the PLLA membranes or polyHEMA hydrogels were incubated in 96 well plates (Corning/Costar Corporation, Cambridge, MA, USA) with culture medium for 24 h at 37 °C. After this incubation, 100 μL of a cell suspension (1.0×10^5 cell/mL) in Ham-F10 medium (Sigma) with 10% FCS (Nutricell) was added to the wells with different substrates. The cells were cultured for 2 h at 37 °C, washed twice with 0.1 M phosphate buffered saline (PBS), pH 7.4, at 37 °C, and then received 100 μL of medium without FCS and with 10 μL of 3-(4,5-dimethyltiazol-2-il)-2,5 diphenyl bromide tetrazolium (MTT, Sigma). After 4 h, 100 μL of isopropanol acid (Isofar Ind. Produtos Químicos, Jacaré, RJ, Brazil) was added. The plate was read in a Multiskan Bichromatic Version 1.06 microplate reader at wavelength of 540 nm. As a positive control for adhesion, the culture plate itself (polypropylene) was used, while silicone adhesive membranes (Rhodiastic) served as a negative control. Blanks without cells were also run for the MTT reaction in all experimental conditions (PLLA membranes, polyHEMA hydrogels, negative or positive controls).

Light microscopy

The cells (1.0×10^5 cell/mL) were added to 24 well culture plates (Corning) containing the different biomaterials. After 48 h in culture, the samples were fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer pH 7.2), and stained with cresol violet for morphological analysis. As a control, cells were also cultured on glass coverslips. The cytotoxicity of the substrates was assessed by direct/indirect contact method [19, 22] in which a glass coverslip was introduced wells containing the different biomaterials and the signs of cell degeneration then assessed. All experiments were done in triplicate. The cells were observed using an Olympus IX-50 inverted microscope.

Scanning electron microscopy (SEM)

For morphological analysis of the biomaterials, samples of PLLA and polyHEMA hydrogels were fractured by immersion into liquid nitrogen. The sample surfaces were gold sputtered and observed in a JEOL JXA-840 scanning electron microscope. For cell morphological analysis of cells, 1.0×10^5 cells/mL were inoculated onto PLLA membranes or polyHEMA hydrogels in Ham F-10

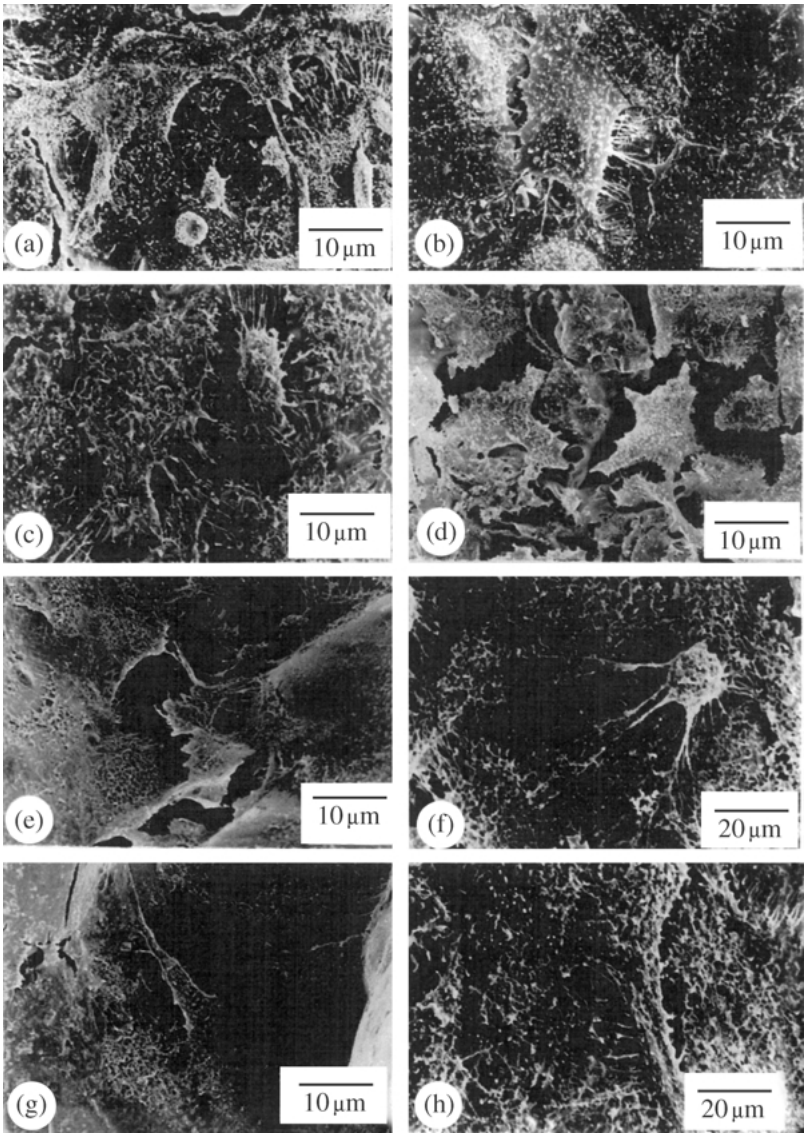

Figure 3 Scanning electron microscopy of Vero cells grown on different biomaterials. (a) Cells grown on a glass coverslip (positive control), (b) polyHEMA with a dense membrane surface, (c) dense PLLA membrane, (d) PLLA membrane with pores $< 45\,\mu m$ (e) and (f) PLLA membrane with 180–250 μm pores, (g) and (h) PLLA membrane with 250–350 μm pores. Barr: 10 μm.

medium (Sigma) containing 10% FCS (Nutricell). Cells cultured on glass coverslips under the same conditions were used as a control. After 24 h, the samples were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M phosphate buffer pH 7.2, for 45 min at 4 °C, and postfixed with 1% OsO_4 (Sigma) in the same buffer for 2 h, at 4 °C. The specimens were then dehydrated through an ethanol series, critical point dried and gold sputtered. The samples were examined in a JEOL 300 scanning electron microscope.

Results

Cell adhesion

The adhesion of fibroblastic cells was greater in the polyHEMA hydrogel than on PLLA membranes. In all cases, the adhesion to the biomaterials was lower than for polypropylene, the positive control (Fig. 1). Fig. 1(a) shows the cell adhesion values for hydrogels with different negative charge densities. The adhesion decreased as the negative charge density increased. Cell adhesion to porous PLLA membranes is shown in Fig. 1(b). There were no significant variations in the pattern of cell adhesion on dense and porous PLLA membranes.

Scanning electron microscopy of different substrates

Fig. 2 shows the typical morphology of the surface of polyHEMA hydrogels and of dense or porous PLLA membranes in SEM. The hydrogel surface was dense and smooth (Fig. 2(a)) while PLLA membranes had a rough, irregular surface (Fig. 2(b)). The morphology of the porous PLLA membranes is shown in Fig. 2(c)–(e). These membranes had a porous structure with different pore diameters, i.e. pore diameter $< 45\,\mu m$ (Fig. 2(c)), diameter 180–250 μm (Fig. 2(d)) and diameter of 250–350 μm (Fig. 2(e)).

Scanning electron microscopy of Vero cells grown on the different substrates

Vero cells formed a semi-confluent layer on glass coverslips. These cells commonly had an elongated morphology with microvilli and/or cell prolongations on

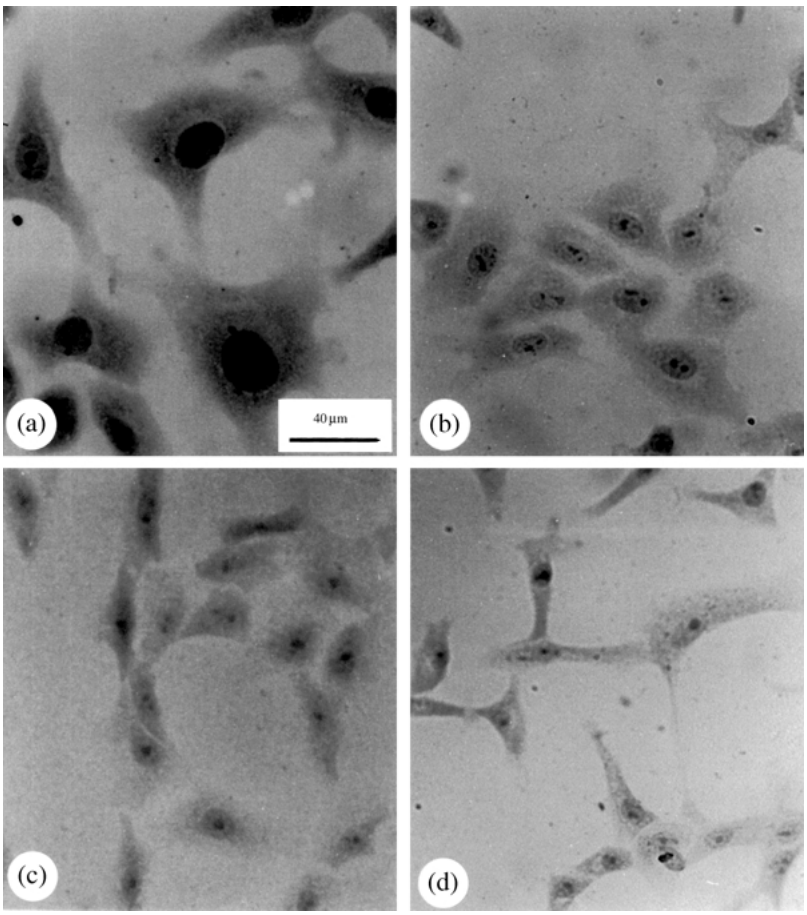

Figure 4 Light microscopy of Vero cells grown on hydrogel membranes of different negative charge densities. (a) Control cells grown on glass coverslips; (b) polyHEMA (0.017 mEq/g); (c) polyHEMA-CA (0.02 mEq/g) and (d) polyHEMA-(MMA-co-AA) (0.11 mEq/g).

their surface (Fig. 3(a)). Cells cultured on polyHEMA hydrogels (Fig. 3(b)) and dense PLLA membranes (Fig. 3(c)) showed a less elongated morphology, but still had microvilli and/or vesicles on their surface. In both cases, flattened cells with cytoplasmatic prolongations that joined adjacent cells were observed. Flattened cells with the largest number of surface microvilli were seen on PLLA membranes with pore diameter $< 45\,\mu m$ (Fig. 3(d)). On PLLA membranes with 180–250 µm pores, the cells had an irregular morphology and numerous prolongations between cells. Often these prolongations formed a thin reticulated network on the cell surface (Fig. 3(e)–(f)). Cells cultured on PLLA membranes with 250–350 µm pores were flattened, with many microvilli and/or vesicles on their surface. Cellular processes linking these cells were also observed (Fig. 3(g)–(h)).

Light microscopy of Vero cells grown on different substrates

Fig. 4 shows the morphological aspects of Vero cells grown on the hydrogels of different negative charge densities. The cell morphology was irregular on the different substrates, and with the cells tending to be more elongated on more negatively charged hydrogels. The morphology of Vero cells on PLLA membranes is shown in Fig. 5. Assessment of cell morphology on this substrate was poor because the material was not translucent. No signs of cell death were observed in cells grown on polyHEMA or PLLA.

Discussion

The applications of biomaterials capable of directing cell behavior are widespread and include devices for tissue replacement and regeneration, as well as substrates for cell culture [2]. The first step in the interaction of the cells with a biomaterial is adhesion. All the biomaterials used in this study showed a limited capacity for stimulating cell adhesion when compared to the positive control used (polypropylene). The low adhesion seen with the different substrates was not caused by any toxic effect of the biomaterials, us demonstrated by light microscopy.

Cell adhesion is modulated mainly by adhesion molecules and specific receptors on cell membranes. On a synthetic surface, cell adhesion is the consequence of protein adsorption to the substrate, and involves many factors, including dipole and electric charge interactions, hydrogen bonds, electrostatic forces, the hydrophilicity/hydrophobicity or surface free energy, and the roughness and rigidity of the surface [10, 11, 18, 24]. PolyHEMA hydrogels were more adhesive than dense PLLA membranes. Hydrogel have low surface tension values, high wettability, and a smooth surface, whereas the surface of PLLA membranes is irregular and rough with a globular morphology and concavities. These characteristics could explain the preference of cells for hydrogel surfaces compared to PLLA membranes. Another explanation for the low cell adhesion to PLLA, may be related to this materials hydrophobic surface which tends to present cell adhesion, in contrast to hydrophilic substrates [4, 5, 7].

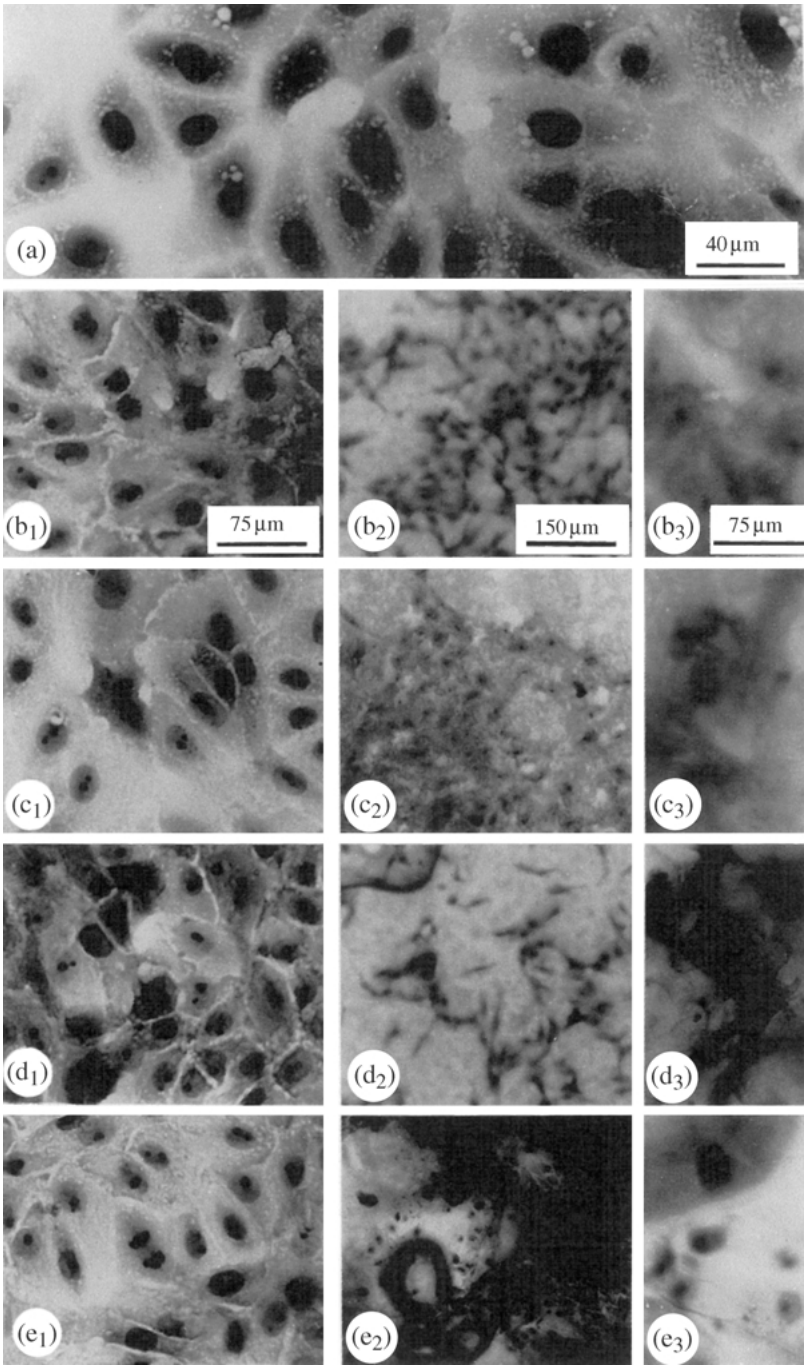

Figure 5 Light microscopy of Vero cells grown on PLLA membranes. (a) Cells grown on a glass coverslip (positive control), (b) dense PLLA membrane surface, (c) PLLA membrane with pores $< 45\,\mu m$, (d) PLLA membrane with 180–250 µm pores and (e) PLLA membrane with 250–350 µm pores. In all cases, b_1, c_1, d_1, and e_1 show the indirect cytotoxicity. The other samples indicate direct cytoxicity. No signs of cell degeneration were observed with any of the substrates. Barr: 10 µm.

Some studies have shown that porous materials can to stimulate cell proliferation and the synthesis of extracellular matrix components. The uniformity of the distribution and the interconnectivity of the pores are important aspects in faciliting tissue formation in an organized network such as used to repair certain types of damaged connective tissues. Variations in pore diameter could make cell substrate interactions difficult by reducing the area for cell attachment, and thus reducing cell adhesion. Although there are reports of cell behavior on large porous PLLA membranes, the influence of variation in the pore diameter in porous PLLA devices has not been described. As shown here variations in the pore diameters of PLLA devices did not significantly alter adhesion pattern of fibroblastic cells.

Resorbable PLLA membranes have been in odontology, especially in periodontal disease. Membranes of this material can promote guided tissue growth leading to bone repair [6]. Other uses of resorbable devices based on PLLA include the adsorption of proteins to stimulate cell growth and differentiation following polymer implantation *in vivo*. Lee and collaborators [25] used a poly(lactic acid-co-glycolic acid) [PLGA] matrix to study the adsorption of a recombinant human bone morphogenetic protein (rhBMP-2), a protein able to promote osteogenesis *in vitro* and *in vivo*. The rhBMP-2-PLGA matrix stimulated new bone formation in bone fractures and improved the repair of damaged tissue. The limitation of this approach was the size of the bone injury [25].

Electrostatic interactions between cells and the substrate are an important mechanism of adhesion since most plasma membrane glycoproteins are negatively charged [26, 27]. For polyHEMA hydrogels, the negative charge density of the material influenced cell adhesion such that increasing the negative charge density of the material surface decreased the cell adhesion and spreading. These results agree with other reports indicating that positively charged materials are better for stimulating adhesion, spreading and cell growth [17, 18, 28]. In the mixed polyHEMA-poly(MMA-co-AA) blend, the $COO^- H^+$ groups of acrylic acid are ionized in the presence of culture medium to provide fixed negative charges in the hydrogel macromolecular network. This characteristic decreases cell adhesion.

The results of this study confirmed the influence of substrate surface properties such as porosity and negative charge on cell adhesion. Although the biomaterials used did not stimulate cell adhesion, the cells were still capable of proliferating (data not shown). Low adherent polymers are not necessarily useless devices and in some cases, a low cell interaction is a desirable characteristic. Thus, low adhesive substrates are essential for maintaining differentiated chondrocytes *in vitro* [29, 30]. The presence of $COO^- H^+$ groups in some biabsorbable hydrogels can decrease macrophage interactions and this may be useful in some clinical applications [16, 31, 32]. In addition, low adherent materials stimulate extracellular matrix production whereas this ability is reduced in highly adherent polymers. In this context, fibroblastic cells cultured on dense or porous PLLA membranes can produce extracellular matrix molecules [33]. Since extracellular matrix production is condition for integration of the polymer with the tissue structure *in vivo*, the characteristics of adhesion and extracellular matrix production must be evaluated when seeking to improve the efficiency of material for tissue implantation.

The morphology of cultured cells varied with the substrate. Cells grown on PLLA membranes showed cell processes (which sometimes joined cells to each other) and a great number of microvilli on their surface. These observations are similar to those previously described for Vero cells cultured on PLLA membranes [33, 34]. Cells grown on polyHEMA hydrogels showed a flattened morphology, with microvilli and vesicles on their surface. This behavior was similar to that previously described by Lombello *et al*. [28, 35]. Alterations in cell morphology are related with the organization of the cytoeskeletal network. The topography of polymers and their surface flexibility are related to cell migration and the functional activity of cytoskeletal proteins [24, 36]. Thus, the physical characteristics of a substrate influenced the morphological alterations observed.

Cells grown on different polymers formed a confluent monolayer. This behavior was similar to that of cells cultured on glass surfaces [37], but differed from the pattern of cell growth on biological substrates, such as three-dimensional type I collagen gels [38] or dry collagen I sponges [39] in which the cells showed a multilayered organization. Since morphology is related to cell function, our results suggest that cells with an altered differentiation pattern on PLLA membranes and polyHEMA hydrogels may have different functional properties.

Acknowledgments

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