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Improved cellular response of ion modified poly(lactic acid-co-glycolic acid) substrates for mouse fibroblast cells

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ABSTRACT

In this report, the effects of argon (Ar) ion irradiation on poly(lactic acid-co-glycolic acid) (PLGA) substrates on biocompatibility were studied. PLGA scaffold substrates were prepared by spin coating glass surfaces with PLGA dissolved in anhydrous chloroform. Previously, we showed that surface modifications of PLGA films using ion irradiation modulate the inherent hydrophobicity of PLGA surface. Here we show that with increasing ion dose (1×10^{12} to 1×10^{14} ions/cm^2), hydrophobicity and surface roughness decreased. Biocompatibility for NIH3T3 mouse fibroblast cells was increased by argon irradiation of PLGA substrates. On unirradiated PLGA films, fibroblasts had a longer doubling time and cell densities were 52% lower than controls after 48 h in vitro. Argon irradiated PLGA substrates supported growth rates similar to control. Despite differences in cell cycle kinetics, there was no detectible cytotoxicity observed on any substrate. This demonstrates that argon ion irradiation can be used to tune the surface microstructure and generate substrates that are more compatible for the cell growth and proliferation.

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1. Introduction

Many organic based materials possess unique properties, including light weight, strength, biocompatibility, and easy processing behaviors, that make them ideal candidates for use in nanobiotechnology and engineering. Potential applications in biomedicine include scaffolds, surgical sutures, implantable devices, tissue regeneration, and wound dressings. A variety of engineered wound dressings and novel skin substitutes have been developed to support zenografts, autografts, and allografts [1]. However, many result in limited skin regeneration, because of bioincompatibility and the presence of antigens that can trigger immune responses, resulting in slow healing rates and increased scar formation [2]. Biomaterials that better mimic the natural features of skin could provide suitable platforms for cell proliferation, differentiation, and attachment and could be used to accelerate the wound healing process [3].

Poly(lactic acid-co-glycolic acid) (PLGA) is a biodegradable copolymer generated from poly(lactic acid) and poly(glycolic acid) that offers a potential substrate for growing cells for use in regenerative therapies. Recently, 3D printing has been used to generate highly patterned, 3-dimensional PLGA scaffolds for cell growth and tissue engineering [4]. However, unmodified PLGA substrates are poor substrates for cellular attachment and growth because of low surface energy and high hydrophobicity [5]. Enhancing the biocompatibility of PLGA and other substrates has typically relied on combining it with extracellular matrix molecules or other biocompatible materials (e.g. collagen, fibronectin, hyaluronic acid) [6–10]. As an alternative to functionalization through the addition of specific biocompatible molecules, irradiation offers inexpensive and relatively simple approach for modifying polymer substrates at the atomic level.

Different types of irradiation techniques, including gamma irradiation, plasma, electron and ion beam, have been applied to polymers modification [11–15]. Slepicka et al. [14] used plasma irradiation of poly(L)-lactic acid (PLLA) substrates to restructure the surface, improve wettability and generate nanopatterns of defined roughness and dimension. Similarly, gamma irradiation is a common method for sterilization of biomedical materials, but can alter the material characteristics of some substrates at higher doses. Increasing gamma irradiation of polycaprolactone decreases contact angle, changes tensile strength and increases proliferation of fibroblasts cultured on the substrates [15]. Gamma irradiation of PLGA decreases the average molecular weight, increases free radical concentration and alters the kinetics of drug release [16]. Ionizing radiation creates cross-links and/or scission of polymer chains and can alter or introduce impurities in the polymer matrix to tailor surface topography through systematic changes in thermal, mechanical, structural, and electrical properties [17–19]. In this study, effects of argon ion irradiation (170 keV) on surface microstructure

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and hydrophobicity of PLGA films were determined. To test biocompatibility, changes in growth kinetics and viability of mouse fibroblast cells cultured on unmodified and argon irradiated PGLA films (10^{12} to 10^{14} ions/cm^2) were measured.

2. Materials and methods

2.1. Substrates

The PLGA polymer (Purac Biomaterials; Lincoln, IL), consisting of poly(lactic acid) and poly(glycolic acid) components in 50/50 M ratio, had inherent viscosity of 1.05 dl/g. PGLA polymers were dissolved in anhydrous chloroform and used to spin-coat autoclaved borosilicate glass coverslips (12 mm Deckglaser; Carolina Biological; Burlington, NC). PLGA films (~200 nm) were irradiated with argon ions (170 keV) at different doses (10^{12} to 10^{15} ions/cm^2) using 200 keV ion implanter from National Electrostatics Corporation (NEC) housed in University of Houston.

2.2. Structural analysis

Surface microstructure was analyzed using X-ray photoelectron spectroscopy (XPS). XPS measurements were obtained by Physical Electronics Model 5700 using monochromatic AlK_α X-ray source 1486.6 eV operated at 350 W. Contact angle measurements were conducted with sessile water drop method using OCA 15EC contact angle goniometer (Dataphysics; Filderstadt, Germany).

2.3. Cells

NIH3T3 mouse fibroblast cells (CLR-1658; ATCC Manassas, VA) were cultured in NIH3T3 media consisting of Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich; St. Louis, MO), supplemented with 4.5 g/L glucose, 10% heat inactivated calf serum (Hyclone; GE Healthcare Life Sciences; Piscataway, NJ), 2 mM L-glutamine, and 50 U/ml penicillin-streptomycin antibiotics and incubated at 37 °C with 5.5% CO_2. Unless specified, all cell culture reagents and cell stains were from Life Technologies (Grand Island, NY). Coated and uncoated coverslips were irradiated in a biological safety cabinet with a germicidal lamp for 20 min per side prior to each experiment to insure sterility. NIH3T3 cells were harvested by trypsinization, counted in a hemocytometer, plated at 1.0×10^4 cells per well (56.2 cells/mm^2) and incubated at 37 °C in 5.5% CO_2 for 24 to 48 h. The entire experiment was repeated 3 times.

2.4. Growth assays

For in vivo staining, cells were incubated for 10 min at 37 °C in NIH3T3 culture medium containing 11 μM Hoechst 33342, 1.14 μM calceinAM and 1.07 μM ethidium bromide homodimer (Live-Dead Viability Assay, Life Technologies). After replacing staining solution with fresh culture medium, cells adherent to the upper surface of each coverslip were immediately photographed using a 10× objective on an inverted microscope (Olympus, Center Valley, PA) equipped with a cooled CCD digital camera (Rolera; QImaging, Surrey, BC, Canada) and image-processing software (Image-Pro; Media Cybernetics, Bethesda, MD). For each coverslip, the objective was focused on cells located on the surface of the coverslip and images from four non-overlapping fields were captured under both Hoffman illumination (DIC) and epifluorescence (350 nm, 488 nm and 568 nm).

2.5. Cell counts

Cells in each captured image were identified by Hoechst staining of nuclei, counted, and scored as live (calceinAM positive) or dead (ethidium bromide positive). Any cells stained with both calceinAM and ethidium bromide was scored as dead. The area of each image in pixels was calculated using Image J, converted to mm^2 and cell density calculated as mean nuclei per mm^2. The doubling rate of the cells was determined by the change in mean cell density at 24 and 48 h for each condition, using publically available software (*Roth V. 2006* [http://www.doubling-time.com/compute.php]). Statistical comparisons of the mean number of live cells/mm^2 on polymer coated substrates vs. control (glass) at 48 h used T-tests with Holm correction for multiple comparisons [20], with $p < 0.05$ considered statistically significant.

3. Results and discussion

PLGA surface modifications were generated using 170 keV Ar ion irradiation. When argon ions interact with the polymers, the ions lose energy via electron and nuclear collision with the polymer. Based on *Stopping and Range of Ions in Matter* software (SRIM 2013), contributions from the electron and nuclear interaction to the formation of defects are nearly equal at the PLGA surface (dE/dX ~ 320 keV/μm). In our earlier work [17], surface microstructural analyses using atomic force microscopy showed systematic changes in surface roughness and surface topography with increasing irradiation. The surface roughness of unmodified PLGA is 0.74 nm. Irradiation effects were observed even at the lowest argon dose of 1×10^{12} ions/cm^2 and, with increasing irradiation, roughness increased to 1.72 nm at 1×10^{14} ions/cm^2. At the highest tested dose of 1×10^{15} ions/cm^2, surface roughness decreased.

Prior to testing biocompatibility, microstructural evaluation was carried out using X-ray photoelectron spectroscopy (XPS) to measure the C1s spectra of unmodified and argon irradiated PLGA (Fig. 1). The spectrum of the unmodified PLGA film has three carbon peaks at 285, 287, and 289 eV. Based on the chemical structure of the PLGA copolymer, the 285 eV peak shows methyl groups (C—H), the 287 eV peak shows the ester linkage (C—O—C) in the polymer backbone, and the 289 eV peak shows the carbonyl group (C═O) of the polymer. Increasing doses of irradiation resulted in the progressive loss of the peaks for C═O and C—O—C bonds and a corresponding increase in the peak for C—H bonds, reflecting the direct modifications of the polymer by the argon irradiation. At 1×10^{15} ions/cm^2, the major C—H peak was increased whereas the C—O—C and C═O peaks were barely detectible, characteristic of extensive scission of the PLGA polymer.

For successful applications in biomedicine, the degree of hydrophilicity of the PLGA surface is crucial. To assess the wettability of the PLGA surface, we measured the dynamic contact angle in PLGA polymers with or without argon irradiation (Fig. 2). The unmodified PLGA polymer showed a maximum initial contact angle of 75° and a minimum

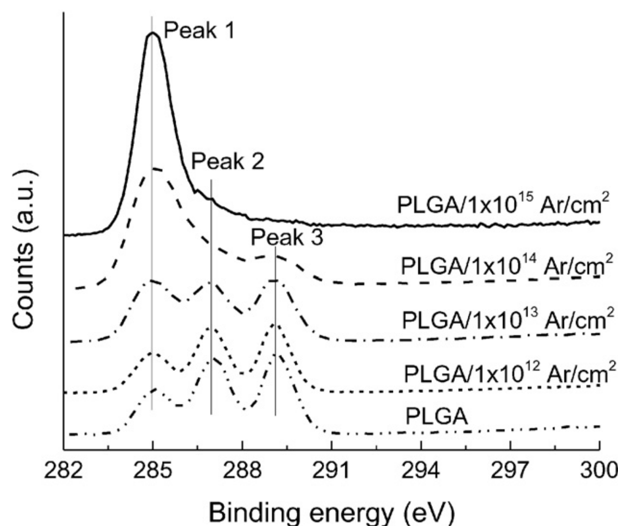

Fig. 1. C 1s spectra of PLGA under different argon ion irradiation where peak 1 (~285 eV), peak 2 (~287 eV), and peak 3 (~289 eV) represents C–H, C–O–C, and C═O bonds respectively.

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