Enhancement of Osteoblastic Bone Cell Proliferation Incubated on Plasma Treated Polymers

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ABSTRACT

It was determined that polyurethane polymers have the potential to be valuable biomaterials. Some major problems with this material involve its low biocompatibility and its inability to induce cellular adhesion and proliferation on its surface, leading to reduced integration in the host osseous system. The efficiency of osteoblast growth on plasma treated polyurethane polymers was tested using separate treatments of nitrogen and oxygen plasma. Bone cells (MC3T3) were cultured on petri dishes coated with a thin film of polyurethane polymer and then plasma treated with either nitrogen or oxygen plasma for three different times for 7 days in comparison to the control samples. This plasma exposure allowed for a greater ease of osteoblast growth than in untreated polymers due to surface roughening at the nanoscale level in a way conducive to osteoblast growth and attachment of additional functional groups to the polymer. Cellular proliferation was analyzed and better proliferation rate was found in the cells grown on the dishes treated for fifteen minutes with nitrogen or oxygen rather than the control samples. Furthermore, it was determined that oxygen plasma treatment is superior to nitrogen plasma treatment. This study indicated that implants coated with plasma treated polymers, particularly those treated with oxygen and nitrogen plasma, can become a reality sometime in the near future.

Keywords: Osteoblasts, Polyurethane polymers, Plasma discharge.

1 INTRODUCTION

Special attention needs to be given to the morphology of the implant surface since this represents the interface between the implant and the host biological system [1]. The essential problem with using bio-implants is their degradation and undesirable biochemical activity with biological tissues adjacent to the implanted material. Considerable efforts have been made to improve the surface properties of metallic implants by designing substrates that are more irregular at the nanoscale, as compared to conventionally used smooth titanium implants surfaces, thus enhancing their ability for osseointegration [2], corrosion resistance [3, 4], and avoidance of infections. Additionally, it is important that these modified surfaces keep the overall valuable characteristics such as biocompatibility and mechanical properties. Increased osteoblast attachment has been shown to take place on surfaces that have been roughened at the nanoscale, compared to smooth surfaces [5, 1].

Pre-osteoblastic cells (MC3T3) have shown good proliferation on implant surfaces coated with biological molecules [6] and exposed to electrochemical treatments [7, 8]. Many approaches such as thermal oxidation, anodization, and sol-gel techniques have been used to minimize corrosion and maximize biocompatibility in implants [9-11], and improving cellular proliferation compared to their untreated control surfaces [6].

In this work, we studied the biologically induced responses and the proliferation of mouse osteoblastic cells over polyurethane polymers and subjected to surface plasma modification. The nanomorphology of these polymeric structures closely mimics the surface topography of bone, similarity with which is a major need for implant surface coatings. We demonstrated that the exposure of the polymers to a plasma discharge of oxygen gas (O2) significantly enhanced cellular proliferation, as compared to the untreated polymeric surfaces and the plastic dishes, then the difference between tow gases (O2, and N2) was studied in details for different time of polymers plasma treatment (5min, 10min, and 15 min). The goal of this study was to prepare superior polymers-based implant coatings by adjusting the structural morphology and introducing chemical modifications onto their surfaces.

The results presented in this paper, further show that not only structural properties of the surfaces, but also the chemical modifications induced by the plasma discharge process, significantly improve the cell proliferation over the polymer surfaces [12, 13]. These results are supported by a combination of techniques that include optical microscopy and the analyzing of the cellular proliferation rates.

2 MATERIALS AND METHODS

2.1 Polyurethane Polymers Coating

Polyurethane has been dissolved in methanol solution for few hours with steering, until w the homogenous solution with out any precipitations was gotten. 35 mm tissue culture plate were coated with the polymers by covering the surface with the liquid and pour the extra materials of the polymers, let the dish upside down to dry for few hours.

The polymeric layer should be homogenous and very thin, after the dryness of the polymers; the dishes were treated with oxygen plasma for different times, and the cells were cultured in a desired density on the polymeric layers and incubated for 7 days. The medium was changed every 48hr.
2.2 Plasma Surface Treatment

Prior to cellular experiments, the polyurethane polymeric films were plasma treated. The plasma reactor used for this process consisted of two parallel aluminum electrodes as shown in figure 1. Plasma was generated between the two electrodes using a 13.56 MHz RF power supply (MCS Plasma Systems Model HF -3) connected to an impedance matching network. The reactor consisted of a unique gas flow design where gas was dispersed into the chamber using a series of inlets from the top electrode. Samples were placed in a ceramic boat between the electrodes and the treatment was done for 15 minutes. The gases were introduced at an operating pressure of 150 mTorr.

![Diagram of plasma reactor](image)

Figure 1. Schematic diagram of the plasma reactor used to plasma treat the polymer surfaces.

2.3 Osteoblastic Cell Cultures Incubation

The murine calvaria-derived MC3T3-E1 osteoblast-like cells were obtained from the American Type Culture Collection (ATCC) and maintained using established procedures. Cell cultures exhibiting exponential growth phase were normally grown in 75 cm² flasks (density of 10^5) with phenol red free alpha-modified minimum essential medium containing 10% fetal bovine serum (FBS), 1% penicillin (500 units/ml) and streptomycin (500 units/ml) at 37°C in a 5% CO₂ atmosphere for 7 days prior to seeding onto each condition, then subcultured by trypsinization for further experiments. The cells were kept in aseptic conditions and the medium was changed every 2-3 days. Cells were seeded at a density of 25x10⁵ cells in 35mm dishes over samples with four different surface treatments in triplicate experiments; the samples were commercially available plastic petri-dishes (untreated), polyurethane polymer untreated dishes, oxygen and nitrogen plasma treated polymers coated dishes. The cells were incubated for 7 days prior to cell proliferation analysis; the medium was replaced with fresh solution every 2 days. All treatments, including the control, were performed in triplicates.

2.4 Cell Assessment with Light Microscopy and Scanning Electron Microscopy

For microscopic studies cells were grown as previously described. The cells were washed thoroughly with 10 mM phosphate buffered saline (PBS, pH 7.4) three times and fixed with 10% formaldehyde solution for 10 min, washed three times with PBS and stained with ethidium bromide-acridine orange fluorescent dye.

The cells were observed with UV light by light transmission microscopy using an Olympus BX 51 microscope. Scanning Electron Microscopy (SEM) images of the TiO₂ substrates were obtained using a JEOL 7000FE.

2.5 Cell Proliferation Analysis

Cell proliferation and viability was analyzed by using a well established Trypan blue assay [14]. First, the cells were cultured as described above for 7 days under various conditions. The cells were then dissociated from the bottom of the plates by trypsinization and transferred to 1.5 ml eppendorf tubes. The solutions, including the detached cells, were centrifuged at 1000 rpm for 10 min and removed by suction. The detached cells on each sample were stained with 100 µL of 1X Trypan blue dye with 1X PBS trypan blue dye (Invitrogen) at room temperature for 5 min. 10 µL of the stained cells were transferred to a hemacytometer and counted using an optical microscope. The data were analyzed by one-way analysis of variance (1- way ANOVA) at the level of p < 0.05.

3 RESULTS AND DISCUSSIONS

3.1 Cellular Proliferation of Plasma Treated Polyurethane Polymers Structure

The effect of the plasma treatment on the proliferation of MC3T3-E1 cells plated on polymer coated plates was investigated and was compared with the control samples composed of untreated polymers and commercial plastic petri dish surfaces. The cellular proliferation results are shown in Figure 2. Osteoblast cells growth on commercial plastic petri dishes and untreated polymers showed a significantly lower proliferation rate compared to the polymer samples treated with O₂ for 5 min. At the end of the incubation time (7 days), the increase in cellular proliferation on the gas (O₂) plasma treated polymers was statistically the most significant (4.72 X 10⁶ cells / well) compared to the polymers untreated surfaces (0.1 X 10⁶) and in the plastic Petri dishes control (1.19 X 10⁶ cells / well). Moreover the cellular proliferation rate have shown a significantly higher level with the cells incubated for 5, 10, and 15 min O₂ plasma treated polymers (4.7 X 10⁶, 5.0 X 10⁶, and 6.3 X 10⁶) as compared with the cellular proliferation rate on N2 plasma treated polymers (0.5 X 10⁶, 0.54 X 10⁶, and 0.62 X 10⁶) respectively.
Figure 2. Cellular proliferation results (a) The cells were plated on different substrates. The plastic dishes, polyurethane polymers, and 5 min O₂ plasma treated polymers, and (b) The cells were plated on the polyurethane polymers O₂, N₂ plasma treated for 5, 10, and 15 minutes. The cells were incubated at a density of 25X 10⁶/well for 7 days at 37°C, 5% CO₂ in humidified incubator, and the growth medium was changed every 2 days.

The assessment of osteoblast cellular proliferation over the various substrates using optical microscopy is shown in figure 3., for this study, the cells were treated with ethidium bromide - acridine orange fluorescent dye and were visualized under UV radiation. The results indicate clearly that plasma treated surfaces induced a significantly higher cellular proliferation, which suggests a better biocompatibility of the surfaces toward the bone cells used in this study.

The aim of this work was to investigate the potential for plasma treated polymer surfaces to enhance osteoblastic proliferation in vitro and to evaluate the effects of these changes relative to the tissue-implant interface [14]. We first compared the proliferation rates of bone cells and then we characterized the most affected surface (O₂ plasma treated polymers samples) in relation to the other surfaces for its responsiveness to the culture conditions. A key finding was that significantly higher proliferation rates were shown after 7 days for the bone cells grown on the O₂ plasma treated polymers for 15 min.

Figure 3: Cellular proliferation analysis. (A) Representative photomicrograph of cultured osteoblastic cells used for these studies. (B) The SEM image of the same cells. (C) Cellular proliferation studies for different substrates and surface modifications: Control (polymeric petri-dishes), Polyurethane polymers as produced (No Plasma) and treated by various gases under the plasma discharge (O₂ and N₂). The cells were incubated for 7 days, stained with ethidium bromide and the images of the cells were captured via Light Transmission Microscopy (10X), (Olympus BX51), (FITC) (green fluorescence).

4 CONCLUSIONS

Development of highly functional and biocompatible coatings for the commonly used metal implants is of a major importance in order to reduce the failure rates of such implants. For such implants to be fully integrated into the host bone tissue successfully, their surfaces must be biocompatible and also promote bone cellular proliferation. We have demonstrated that by altering the nano-morphology of the surface by developing polymers that are treated by plasma gas discharge can result in significant improvement in the proliferation of osteoblast cells when attached to these surfaces. Plasma surface modification can be used to induce a specific surface chemistry without changing the bulk properties of the materials and can be tailored by varying the different operational parameters involved in the process. In our study, the gas discharge with the best effect on cellular proliferation was O₂, a result that can be explained by the formation of oxygen based surface functional groups and atom/ion implantation, which promote cell growth and adhesion.

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