Full Paper

INVESTIGATION OF THE EFFECTS OF LASER MICRO-TEXTURING ON THE CELLULAR RESPONSE OF SILICON SURFACES

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ABSTRACT

This study investigated the effects of laser micro-texturing on the interaction of silicon with cancer cells. This was with a view to improve the biocompatibility of silicon and to enhance its biomedical applications. The modification of silicon surface was carried out by the fabrication of microgrooves of different spacing on its surface using laser nano-second technique. Surface characterizations of the modified and unmodified samples were carried out using Scanning Electron Microscopy (SEM). Human osteosarcoma cells were cultured on the surfaces to study the effects of this modification process on cellular response. Optical and immunofluorescent microscopy were used to study the morphology and adsorption of cells on the different cultured samples. Micro-texturing was found to encourage the spreading of cells, with definite orientation and alignment along the groove ridges, thereby improving its bio-compatibility. Increasing grooves spacing was found to enhance the adsorption and proliferation of cancer cells. This study therefore facilitates the development of substrates with improved biocompatibility for the design and fabrication of biomedical devices for cancer diagnosis and treatment.

Keywords: Laser micro-texturing, microgrooves, surface modification, cellular response.

1. INTRODUCTION

The interactions of materials with biological cells are initiated at the surface and this have been found to either impair or enhance ability of the implant, or device, to integrate and operate within the biological system in which it is placed (Williams, 1987). The attributes of implant surface such as surface tension, surface topography and surface chemistry have been established to influence these interactions. Several researchers have therefore carried out extensive study towards enhancing cellular response on biomaterials surfaces (Smith 1994, Richards, 1996, Brunnette and Chehroudi, 1999, Baseter *et al*., 2002, Lee *et al*., 2002, Price *et al*, 2003, Nayab *et al*., 2003, Recknor *et al*., 2004, Blazewiez *et al*., 2004, Rao *et al*., 2018, Chakraborty *et al*., 2019 and Zafar *et al*., 2019). One of the well reported means of enhancing cellular response is the modification of surface attributes to yield desired attributes that will improve the biocompatibility of the substrate (Bhalerao *et al*., 2004, Milburn and King, 2004, Mwenifumbo *et al*., 2007, Pfleging *et al*., 2015, Rao *et al*., 2018, Chakraborty *et al*., 2019, Zafar *et al*., 2019).

Generally, modification of surface characteristics such as, topography, surface chemistry and surface energy have been found to play crucial roles in cell adsorption, adhesion and proliferation (Nayab *et al*., 2003, Blazewiez *et al*., 2004, Lim *et al*., 2004, Milburn *et al*., 2009 and Zafar *et al*., 2019). Furthermore, enhanced biological responses such as biostability, biosafety, bioactivity and biocompatibility have also been reported to be influenced by surface modifications (Chen *et al*., 2009, Rao *et al*., 2018, Chakraborty *et al*., 2019, Zafar *et al*., 2019). Consequently, it is imperative to investigate methods of modifying biomaterial surfaces to facilitate the design and development of biomedical devices for diverse applications. Techniques for surface modifications has been broadly classified into physiochemical and biomolecular techniques (Mwenifumbo, 2004). Physiochemical methods consist of physical alteration of atoms or molecules at the surfaces, which may be accomplished using laser irradiation, plasma techniques, mechanical blasting, spraying, etching or by chemical modification such as coating of substrate with materials of different chemical composition. On the other hand, biomolecular surface modifications involve surface mobilization of biomolecules by physical entrapment, physical adsorption or covalent attachment.

Silicon is usually considered as suitable candidate materials for implantable biomedical devices, such as bioMEM and biosensor, due to its ease of fabrication by micro fabrication techniques and its salient properties (Petersen, 1982). Its use is however fraught with

a number of limitations such as eliciting of cyto-toxicity, high adverse immunological response, and poor cellular response (Mwenifumbo, 2004). Modification of silicon surface is therefore vital to enhance its biocompatibility in service.

Several study investigating the effects of surface modifications on silicon cellular interaction has been well-documented (Milburn, 2004, Mwenifumbo, 2004, Milburn *et al*., 2009). Techniques involving the use of biocompatible coatings, such as titanium, to enhance the biocompatibility of silicon-based device has been reported (Milburn and King, 2004, Oyatogun *et al*., 2013). Similarly, Mwenifumbo (2004) investigated the effects of modification of surface topography of silicon on its biocompatibility and reported that laser microtexturing resulted in enhanced biocompatibility. This research is therefore a follow up on this as it entailed a detailed study of the effects of increasing microgrooves spacing on silicon cell surface interactions.

2. METHODOLOGY

2.1. Sample Preparation:

Silicon wafers obtained from Silicon Valley Microelectronics, San Jose, CA, which consisted of n-type, four-inch single crystal silicon (100) wafers with 375 µm thickness were cut into appropriate dimensions using a diamond scribe. The samples were then cleaned using the standard clean-1 procedure for cleaning of silicon (SC-1). This consisted of removal of organic contaminants by boiling the wafers in a 1:1 H_2SO_4: H_2O_2 solution; removal of metallic contaminants by further boiling in 1:1 HCl: H_2O_2 solution after which the wafers were rinsed in dd water and finally sterilized in ethanol (Oyatogun *et al*., 2013). The silicon samples were then given a final rinse in dd H_2O after which they were sterilized in ethanol. Some of the silicon samples were utilized for the micro-texturing experiment while the others served as the control.

2.2. Laser Micro Texturing:

Laser micro texturing of the surface of some of the silicon wafers were carried out at Spectra Physics Inc., Mountain View, CA, using the Spectra-Physics Navigator II YHP40 laser. This consisted of a modular diode pumped laser head, which produced laser outputs at three wavelengths: 355 nm (UV), 532 nm (Green), and 1064 nm (IR), using interchangeable frequency conversion module. The laser produced pulse repetition frequencies (PRF) in the frequency range of 1 to 100 kHz. Variation of the PRF, scan speed, and wavelength resulted in creation of parallel grooves on the surfaces of the silicon samples. A Scanlab, Hurry Scan Laser Scan Head equipped with a 160 mm telecentric f-theta objective, was used to focus and move the beam to produce microgrooves of different grooves spacing.

In order to remove the SiO_2 deposits and loose particulate formed during the laser irradiation process, the micro textured samples were ultrasonically cleaned in solution of double distilled water (dd water) and detergent for twenty minutes, rinsed in dd water and finally ultrasonically cleaned in acetone for 20 minutes to ensure adequate removal of the submicron particles without causing cavitation damage to the wafer surface. Detailed surface characterization of the silicon sample surfaces was carried out before and after the surface modifications processes using the Philips XL-30 field emission scanning electron microscope (Phillips, New York).

2.3. Cell Culture:

The quality of attachment of cells to the various surfaces was studied by culturing Human Osteosarcoma cells (HOS), obtained from American type culture collection in Manassas, VA, on both the microtextured and control sample surfaces. A cell suspension, with cell concentration of approximately 50,000 cells / mL, was prepared using standard protocol (Oyatogun *et al*., 2013). In order to equilibrate the silicon samples with the cell culture environment, the samples were placed in cell culture wells containing 1.5 mL of serum-free (Dulbecco's Modified Eagle Medium (DMEM) and allowed to incubate for fifteen minutes at 37 ^{0}C. One hundred microlitres of the cell suspension was pipette onto each of the samples' surface and allowed to incubate at 37 ^{0}C for their allotted periods of time; 15 mins, 30 mins, 60 mins 24 hours and 48 hours: Previous work has shown that 60 minutes is sufficient to study the quantity of cells that might have adsorbed on the surface of the samples and that by 24 hours the cells would have spread adequately for the study(Oyatogun *et al*., 2013). Optical microscopy and immuno-fluorescence microscopy were used to study and record the attachment of cells to the various surfaces at the different culture time.

2.4. Optical Microscopy:

The interaction of the surfaces with cancer cells was studied using Nikon 50i research microscope and images were taken using a Nikon Instruments DXM 1200F colour digital camera (Melville, NY). Optical images of the cultured cells on the microtextured surfaces at the different culture time of 15 mins, 30 mins, 60 mins and 24 hours 48 hours were taken. These facilitate a close observation of the cells' attachment and spreading processes on these surfaces. Optical images of the cells cultured on the unmodified silicon sample, at different cultured time were also taken to serve as control for the experiment.

2.5. Fabrication of Machine Parts:

In order to study the quality of cells attachment to the substrates, cells were cultured on the different sample for 48 hours. After the 48 hours incubation period, the specimens were stained with fluorescence-labelled antibodies to reveal cytoskeletal (actin) and focal adhesion (vinculin) proteins. The immuno-fluorescence staining process consist of fixing of the cultured cells on the substrate for 15 minutes in a 3.7% formaldehyde solution. This was followed by preparation of Solution A buffer by diluting 1M $MgCl_2$ solution in Phosphate Buffered Saline (PbS) solution at a ratio of 1: 2000 -solution A was used to dilute most of the reagent used in the course of the staining. The cells were then made permeable on the substrate by exposing them to a 0.5% Triton solution for 15 minutes followed by careful rinsing in solution A and subsequent exposure to primary antiserum, which consists of 1:300 dilution of the primary anti-vinculin antibody in solution A along with 2.0% ovalbumin. Incubation of the permeated cells in the primary antiserum was carried out for 30 minutes in a humid atmosphere at 37 ^{0}C. The samples were subsequently rinsed in solution A and incubated in a secondary antiserum, consisting of rhodamine phalloidin in 1:500 dilution and a secondary anti-vinculin antibody in 1:600 dilution in solution A plus 2.0% ovalbumin. Finally, the samples were incubated while exposed to the secondary antiserum, for 30 minutes at 37 ^{0}C after which a few drops of Fluoro Guard reagent (Bio-Rad Laboratories, Hercules, CA) was added to the seeded micro-textured specimens and cover slips were placed over the specimens.

Immuno-fluorescence microscopy study was carried out using a Nikon 50i research microscope, with an EPI-fluorescence attachment while images were taken using a Nikon Instruments DXM 1200F colour digital camera (Melville, NY).

3. RESULTS AND DISCUSSION

SEM micrographs of the microgroove surfaces are presented in Figures 1. This consisted of the micrographs of microtextured silicon with grove spacing of 20, 30, 40, 50 and 60 µm, Figure 1.0 (a – e) respectively. Figure 1. (a) was taken at magnification of 1820, working distance of 11.2 and scale bar of 20 µm, (b) was taken at magnification of 1957, working distance of 10.7 and scale bar of 10 µm, (c) was taken at magnification of 1521, working distance of 11.2 and scale bar of 20 µm while (d) and (e) were taken at magnification of 2681, working distance of 11.2 and scale bar of 10 µm and magnification of 2158, working distance of 11.0 and scale bar of 10 µm respectively. Finally, Figure 1 (f) shows the SEM micrograph of the unmodified silicon surface taken at magnification of 804, working distance of 11.1 and scale bar of 20 µm.

From these micrographs, it can be observed that the surface roughness of the microgroove samples is increased by the laser micro texturing processes, Figures 1 (d and e). Increasing the groove spacing however resulted in a decrease in the concentration of the micro features, since increasing grooves spacing have resulted in fewer grooves per unit area. Consequently, increase in the surface roughness of the microtextured samples occurred with decreasing grooves spacing Figure 1(a-e).

Surface roughness has been acknowledged as a crucial factor in the promotion of implant integration with biological tissue and the stimulation of the healing process (Matsuzaka *et al*, 2003, Bagno and Bello 2004). The effects of laser micro texturing of the silicon surface on its cellular response can be observed in the optical images of cells cultured on the microtextured surfaces presented in Figures 2 to 6. The numbers of adsorbed cells at 15mins. culture time on the microgrooved surfaces can be observed to increase with increasing groove spacing. Little or no cells were found on the 20µm groove spacing while more cells were found on the 30 to 60 µm grooves spacing, Figures 2 (b - e).

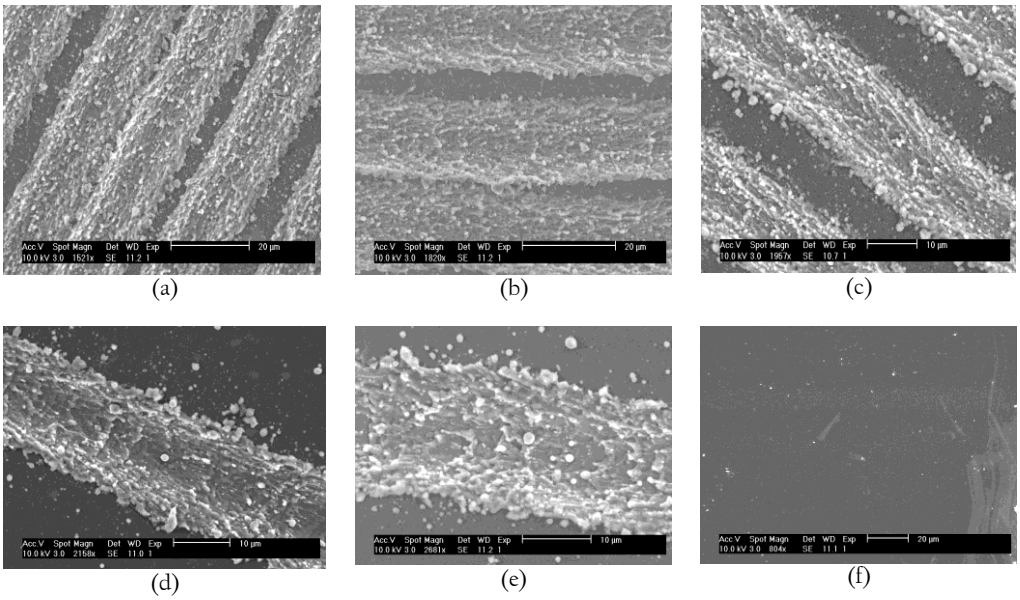

Figure 1. SEM Micrographs of microgrooves and control surfaces showing the effect of increased groove spacing on the material's surface topography: (a) 20µm x 1521, (b) 30µm x1820, (c) 40µm x 1957, (d) 50µm x 2158 (e) 60µm x2681 and (f) Unmodified Silicon x 804

Similarly, proliferation and spreading of cells were observed to increase with increased grooves spacing while formation of filopodia was observed as the cells spread from the spherical shape to a more flattened shape on the micro-grooved substrates as the culture time increased (Figures 5 to 6). This was however observed to be more pronounced in the substrate with increased groove spacing, Figure 6 (c – e) while the attachment and spread of cells on the unmodified silicon samples were found to be more when compared with those on the microtextured samples, Figures 1 to 6 (a). This may be ascribed to the increased surface roughness observed on the microtextured sample. Since increasing groove spacing resulted in fewer grooved per unit area, the surface roughness of the microtextured sample may be deduced to reduce with increasing groove spacing. Consequently, the observed decrease in the quantity of cells adsorbed on the substrate with decreasing grooves spacing may be attributed to increase in the substrate surface roughness.

Furthermore, fewer cells were noticed on the micro-grooved surfaces when compared to the unmodified surface, these were however aligned parallel to the direction of the grooves (Figures 3 to 6) while the cells cultured on the unmodified silicon surfaces were found to be randomly oriented.

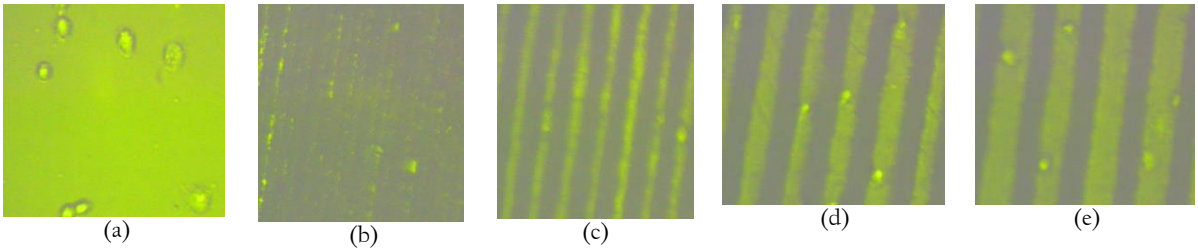

Figure 2. Optical images of cells cultured for 15mins showing the effect of increased grooves spacing on the quantity and quality of cells attachment: (a) silicon (b), (c) (d) and (e) microtextured silicon samples with 20µm, 30µm, 40µm and 50µm groove spacing respectively x 20

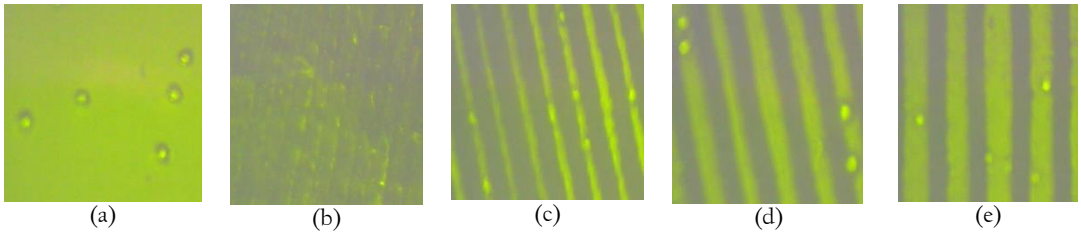

Figure 3. Optical images of cells cultured for 30mins showing the effect of increased grooves spacing on cell spreading at 30mins cell culture time: (a) silicon (b), (c), (d) and (e) microtextured silicon samples with 20µm, 30µm, 40µm and 50µm groove spacing respectively x 20.

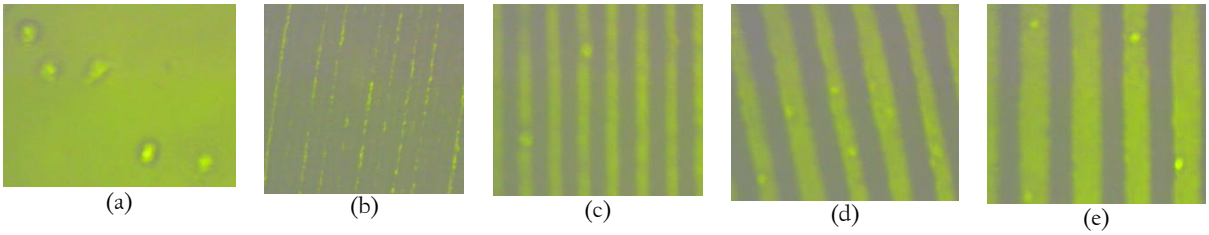

Figure 4. Optical images of cells cultured for 60mins showing the effect of increased grooves spacing on cell spreading at 60mins cell culture time: (a) silicon (b), (c), (d) and (e) microtextured silicon samples with 20µm, 30µm, 40µm and 50µm groove spacing respectively x 20

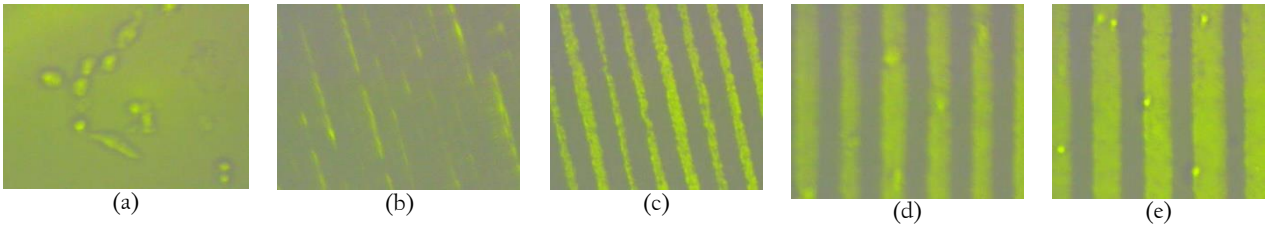

Figure 5. Optical images of cells cultured for 24hrs showing the effect of increased grooves spacing on cell spreading at 24hrs cell culture time: (a) silicon (b), (c), (d) and (e) microtextured silicon samples with 20µm, 30µm, 40µm and 50µm groove spacing respectively x 20

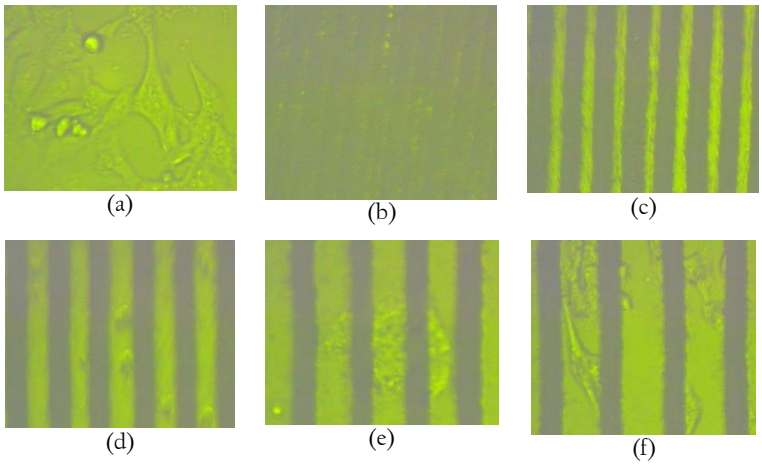

Figure 6. Optical images of cells cultured for 48 hrs showing the effect of increased grooves spacing on cell spreading at 48 hrs cell culture time: (a) Silicon (b), (c), (d), (e) and (f) microtextured silicon samples with 20µm, 30µm, 40µm, 50 and 60µm groove spacing respectively x 20.

From the immuno-fluorescence image of the micro-grooved surfaces (Figure 7), the cells can be observed to be in an elliptical or spindle shape, which are aligned parallel to the groove direction in an end-to-end fashion along the ridges and within the grooves. Andrade and Hlady (1986) had previously suggested that cell alignment within grooves may be based on thermodynamics and extra cellular matrix (ECM) protein adsorption. According to the authors, edges of grooves differ in reactivity from nearby planar surfaces due to the existence of more valences and unsaturated bonds at the edge, which they suggested may alter the ECM proteins involved in adhesion. Similarly, surface topography, especially grooved surfaces, has been shown to play crucial roles in cellular response to oral implants (Matsuzaka *et al.*, 2003). Furthermore, a number of studies confirming the influence of microtextured surfaces on cellular behaviour in both a quantitative and a qualitative manner has been reported (Den-Braber *et al.*, 1995, Den-Braber *et al.*, 1996, Walboomer *et al.*, 1998, Walboomer *et al.*, 1999, Matsuzaka *et al.*, 1999, Wang *et al.*, 2000, Walboomers and Jansen, 2001, Matsuzaka *et al.*, 2003, Mwenifumbo 2004, Milburn, 2004, Milburn *et al.*, 2009). One of the researchers, Mwenifumbo, 2004, reported that cells cultured on polished and blasted surfaces were randomly oriented, while those cultured on the micro-grooved

surfaces exhibited contact guidance. Most of the work carried out by other researchers confirmed this finding (Brunette, 1988, Brunette *et al.*, 2001, Soboyejo *et al.*, 2002, Matsuzaka *et al.*, 2003). The results obtain in this study was therefore in conformation with what had been previously reported and micro-texturing of the silicon surface have consequently been found to enhance cellular response.

In addition, results obtained from the Immuno-fluorescence staining confirmed changes in the internal organization of the cells associated with contact guidance along the grooved ridges. On the micro-grooved surfaces, the alignment of actin microfilament, visible as red on the immunofluorescence image, reflected the orientation of the cells (Figure 7). Matsuzaka *et al.*, 2003, ascribed this to the anisotropic geometry of the grooves and ridges, which establish stresses and shear-free planes that influence direction of stress fibre growth. Furthermore, after 48 hours, thin dense vinculin positive patches (green) were present on all the investigated surfaces.

The mode of adhesion of the HOS cells on the different surfaces is illustrated by the distribution of focal contacts of the cells on the substrate. On the smooth surfaces, the focal contacts were observed to be distributed uniformly on the membrane surfaces that were in contact with the substrate. On the micro-grooved substrate, focal contacts were visible on the extremities of cell extensions where the cell membranes were in contact with the substrate. Co-localization of actin and vinculin showed that the actin formed numerous stress fibres throughout the cell, which terminated at the cell periphery. It also showed that the vinculin-containing adhesion plaques were situated at the terminal ends of the actin stress fibres. Subsequently, we may infer that although the quantity of cells cultured on the micro textured samples is few, when compared to those cultured on unmodified silicon samples, they had a definite alignment along the groove ridges with cells that were observed to migrate and spread along these ridges in a particular direction, as shown in the immuno-fluorescence images of the microgroove samples.

The adoption of this surface modification process will therefore improve silicon biocompatibility. This possibly will improve its cell surface integration and may minimize the occurrence of adverse host response such as biofouling, cyto-toxicity, fibrous encapsulation and subsequent rejection by the host system.

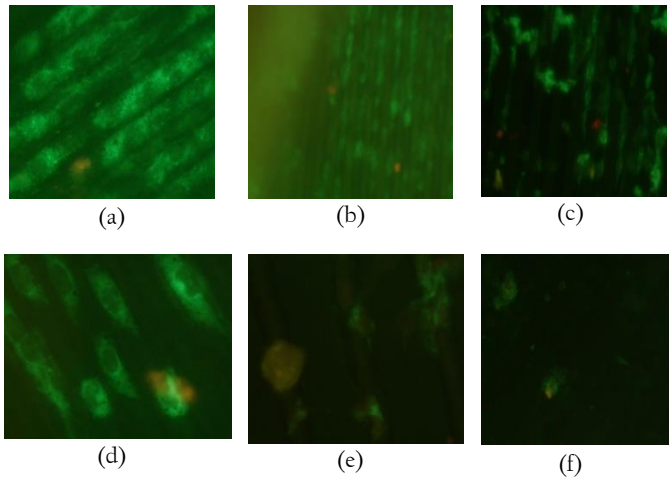

Figure 7. Immunofluorescent images of cells cultured for 48 hrs showing the effect of increased grooves spacing on cellular interactions: (a) 20μm, (b) 30μm, (c) 40μm, (d) 50 μm, (e) 60 μm micro grooved silicon Samples and (f) Control silicon sample x 60.

4. CONCLUSION

Laser micro texturing of silicon surface has been found to result in the attachment and spread of cells with definite orientation and alignment. This therefore showed that micro-texturing could be depended on for enhanced cellular interaction and consequently improved biocompatibility. However, the degree of alignments was found to be impaired with increasing grooves and grids spacing, hence, lower grooves spacing is recommended for enhanced cellular response.

Laser micro texturing of silicon surface will therefore facilitate the development of substrates for the design and fabrication of biomedical devices for cancer diagnosis and treatment.

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