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Laser Surface Modification of Poly (ε-caprolactone) Scaffold for Artificial Skin Applications

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Abstract

Hard implants undergo detachment from the host tissues due to inadequate biocompatibility, poor adhesion and subsequent cell integration. Thereby, surface engineering seems to offer solutions for improved functionality and biocompatibility of material implants inside the biological environment. Polycaprolactone (PCL) thin film has been fabricated via uniaxial compression technique. Pulsed excimer laser was used to modify the PCL surface roughness. The laser pulses induced the formation of nanoripples on the membrane surface. The effect of laser parameters (pulse rate, energy and number of pulses) on the development of the nanoripples was studied. The surface morphology, roughness and the scaffold biocompatibility and cell viability were characterized using scanning electron microscopes (SEM), atomic force microscope (AFM) and MTT assay micro-plate reader. This work showed that applying laser pulses at different rates significantly modified the surface criteria. The modified scaffold was more biocompatible, rough, with enhanced cell attachment, proliferation and provided adequate host for cells to differentiate rather than the unmodified scaffold. The results clearly revealed that the modified scaffold with nanoripples on its surface could be a candidate implant material for artificial skin applications.

Keywords: Surface engineering, PCL, Surface roughness, Laser.

1. Introduction

Recently, Tissue engineering (TE) is gaining lots of attention as an emerging discipline that is advancing rapidly [1]. Medical applications can greatly benefit out of this development as tissue engineering represents a good alternative for biological substitutes in medical applications [2]. TE holds promises of eliminating re-operations by using biological substitutes [3], solves problems of implant rejection [4], transmission of diseases associated with xeno-grafts and allo-grafts, and shortage in organ donation [5-6]. Furthermore TE provides long-term solutions in tissue repair [7], and potentially offers treatments for medical conditions that are currently untreatable[8-11]. Development of suitable biodegradable materials and scaffolds for seeding cells is the key of tissue engineering. Developing scaffolds is very challenging as they should be biocompatible[12], biodegradable [12] and provides good cell adhesion [13]. Tissue engineering is critical in need to ideal scaffold material and design [13-15]. Scaffolds can be prepared by either chemical or physical techniques. Chemical techniques include foaming[16], freeze drying[17] and phase separation[18]. Whereas, physical techniques are based on laser sintering[19], and rapid prototype machining [20]. There are lots of current limitation for the chemical methods due to the high impurity, lack of producing ideal scaffold for in-vivo applications[21]. On the other hand, physical treatments to the surface using laser ablation [23], plasma [24] or UV photo-curing [22] seems to offer solutions for improved functionality and biocompatibility of material implants inside the biological environment[23].

The laser-assisted modifications of polymer surfaces offer advantages over both chemical and other physical methods. They enable precise modification of certain surfaces that are difficult to be treated with conventional other methods. The resulting modified surfaces are free from contamination. They also could improve functionality and biocompatibility of materials which subsequently could be active implants inside the biological environment [24-28].

Synthetic and natural polymers are used for drug delivery [29,30,31] and tissue engineering applications[32]. One of the synthetic polymers is PCL which posses many advantages. PCL is semi-crystalline linear resorpable aliphatic polyester[33]. It undergoes biodegradation[34] due to the susceptibility of its aliphatic ester linkage to hydrolysis. At present, PCL is regarded as a soft and hard tissue compatible bioresorpable material and it has been considered as a potential substrate for a wide spectrum of applications, such as drug delivery systems[35], bone tissue engineering [36], engineered skin[37], axonal regeneration, and scaffolds for supporting cell growth such as fibroblasts and osteoblasts [38-40].

Herein, the surfaces of PCL scaffolds have been modified via laser treatments with varying the laser parameters such as energy, pulse rate and number of pulses. The effects of these parameters on the surface morphology, cell viability and differentiation was studied and characterized by SEM, AFM and MTT assay micro plate reader.

2. Experimatal setup

2.1 Materials

Poly (ε-caprolactone) (average molecular weight (~Mw) 60,000), Dulbecco's Modified Eagles Media (DMEM), glutamine, penicillin, Streptomycin, Ethanol HPLC and MTT dye [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were provided by Sigma Aldrich, Chemie GmbH (Munich, Germany). Phosphate buffer saline (PBS) and Polycarbonate filter paper were provided by El-gomhoria Co. Cairo, Egypt. Double deionized water from a Milli-Q™ system (Direct-Q 3, Model ZRQS0P0WW, Millipore Corporation, Billerica, MA) with a resistivity of 18 MΩcm. All purchased Chemicals were used without additional purification.

2.2 PCL membranes preparation

PCL membranes have been fabricated by uniaxial compression technique; briefly PCL granules were heat pressed in a steel mould covered with a copper sheet (in order to equalize

heat distribution) using a heat controlled piston at 60°C for 30 minutes. Then the steel mould was quenched in iced water to obtain a compressed and free of bubbles PCL bar with a dimension of (3×5×20) mm. The PCL bar was heat pressed and quenched subsequently in iced water in a glass mould with the previous conditions to produce a PCL membrane of a thickness of 0.1 mm.

2.3 PCL scaffold modification with excimer laser

Excimer laser pulses have been generated from a Lambda Physic Compex 205 system having krypton fluoride (KrF) as the lasing gas, resulting in a laser wave length of 248 nm, a band width of 300 pm and a pulse duration of 24 ns. The output pulse has a rectangular transverse cross section with a width of 0.6 cm and a height of 2.4 cm. The beam intensity has a gaussian distribution in the vertical and horizontal directions. A beam guiding system has been used to reshape the pulse wave front into a square of 1.6 × 1.6 cm2 and to homogenize the intensity of the beam in the transverse direction. The beam spot size on the target is 0.48 × 0.48 cm2. The pulse energy density has been varied from 15 to 25 mJ/cm2, the number of pulses has been changed from 500 to 2500 whereas the repetition rate has been changed from 2 to 5 Hz. The treatments have been conducted under standard atmospheric conditions.

3. Characterization of the scaffold

3.1 SEM imaging

The microstructure and morphology of the scaffold have been examined using field emission scanning electron microscope (SEM, LEO SUPRA 55; Carl Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 8-12 kV (SEM) and with magnification in the range of 50-400 Kx. Briefly the samples were bleached, mounted on carbon tab and coated with gold using coated sputter (S150A Edward, England) and examined.

3.2 Cell scaffold interaction

Modified and unmodified PCL films were cut into 0.7 inch diameter circles. One film was cut to about 9 small substrates. Scaffolds of size 7mm × 7mm were seeded in culture medium with human Fibroblast cells in 24 well plates for a period of 14 days. Briefly the scaffolds were submerged in 70% ethanol for 15 minute, sterilized with carbon dioxide at 50ºC for one hour. They were then washed with PBS (7.4) and were soaked in 5ml of culture medium for 1 h. The cells were grown in the plates at a density of 5×106 cells/ml in Medium (DMEM) containing fetal bovine serum (10%V/V) glutamine (2 mM), penicillin (100 U/ml) and streptomycin (1 μg/ml) and incubated in 5% CO2 at 37°C. The culture media were changed every 48 hours for a period of 14 days. The experiment was repeated 3 times to estimate the standard deviation.

3.3 In-vitro biological testing imaging

The cell viability, cell proliferation and cell adhesion were evaluated via scanning electron microscope. The cell seeded scaffolds on days 3, 7, and14 were prepared for SEM observation by changing culture medium, washing 3 times in prewarmed PBS (7.4). After washing with PBS, the specimens were fixed in 2.5% glutaraldehyde at 40C for 4 hours and dehydrated in ascending grades of ethanol changes (50, 70, 90 and 97%), each step lasting 30 min. Then the samples were left to dry for two hours , mounted on aluminum stubs coated with carbon tape and examined by environmental scanning electron microscope under 6-10 kV (SEM).

3.4 Roughness determination

The surface roughness of the scaffolds has been examined and measured using atomic force microscope (Veeco Metrology, Plainview, NY, USA).

3.5 In-vitro degradation test of the scaffold

In-vitro degradation of the scaffolds was investigated by soaking in simulated body fluid (SBF) (PH 7.4) at 370C for 10 days. The ratio of solution volume to scaffold mass was 200 ml/g. After soaking, the scaffolds was dried at 30ºC for 1 day and the rate of weight loss of the scaffolds with different PCL contents as a function of soaking time in SBF were measured. The

experiment was repeated 3 times to estimate the standard deviation.

4. Results and Discussions

4.1 SEM imaging of the scaffold

The morphology of the modified and unmodified PCL scaffold was shown in Figure 1 (A-F). In Figure 1(A-B) the surface of the scaffold was modified by changing the pulse rate (PR) 5Hz (A) and 3Hz (B). The varied PR lead to the formation of highly distributed, regular ripples on the scaffold surface with size ranged from 50-200nm in width and extended to 0.6-2µm in height. In Figure 1 (C-D) the surface of the scaffolds were modified by varying the number of pulses particularly 500(C) and 1500 (D). The ripples obtained in this case had size in the range from 20-150nm in width and 200-800nm in length. The ripples obtained by changing the energy density in the range of 20mJ/cm2 were shown in Figure 1 (F). They had size ranged from 200-500nm in width and 1-2µm in length. In contrary, the surface had rough surface with rippled ripples with lack of deep ripples by using energy density in the range of 25mJ/cm2 Figure 1 (E). The SEM results revealed that the surface of PCL gained highly ordered, distributed ripples by changing the laser parameters particularly (energy density, pulse rate and number of pulses). In addition, the pulse rate had the greatest effect on the surface morphology. This data was in agreement with the data reported before for surface engineering via laser ablation and its ability to change surface morphology [41-42]. In the present study, the effect of excimer laser on the surface morphology have been studied to obtain the best rough surface to be used as a substrate for artificial skin.

Formation of these ripples in this study could be attributed to the fact that using a short pulse, medium laser intensity and lower number of pulses results in disintegration of PCL scaffold at a much faster rate and before the melt could land on the membrane surface that have already been disintegrated by the oncoming pulses [43]. In the modified scaffold by laser ablation, energy has to be delivered in excess of the binding energy of the atom so as to remove the atom from the solid or change chemical composition of these atoms. PCL melting point is very low (65ºC), thus, to modify the surface of the scaffold via changing the chemical composition we applied a short pulse, medium laser intensity, and this fluence is approximately inversely proportional to the pulse duration[44]. The fluence is able to ionize almost any target material that takes place early in the laser pulse time. Following ionization, the laser energy is absorbed by free electrons[45]. These energetic electrons break free from the bulk material and create a strong electric field due to charge separation from the parent ions. The magnitude of this electric field pulls the ions out of the solid target when the electron energy is larger than the binding energy of the ions, thus resulting in material removal[46]. In laser ablation of the scaffolds, the heat diffusion into the scaffold material is not negligible and the energy loss into the sample is minimized. There were many factors that could affect the result of the ripple perforation. First, the pulse energy (PE) of the laser could directly affect the size of the ripples[47-48]. A higher PE generally leads to scaffold melting and form larger craters. Next, the pulse number (PN) can affect the quality of the produced ripples, as low pulses generate greater ejections of melt on the scaffold, which subsequently leads to higher melting degree of the scaffold. It is clearly indicated that the PCL scaffold with ripples perforated at lower pulse numbers and had higher surface energy. At lower pulse numbers, the degree of surface roughness is higher because there were more surface asperities due to greater splattering effect. These asperities can influence the water droplet and particle interaction forces by modifying the Van der Waals interaction, which would then rupture the liquid membrane that holds the liquid together[49]. This rough surface is more suitable for cell growth and proliferation when implanted in the organs[45].

4.2 Roughness analysis

The average plane roughness of the scaffolds surface was monitored by AFM following excimer laser modification by changing the parameters (pulse rate, number of pulses and energy density). The surface roughness had

values in the range of 150-320 nm by changing the number of pulses particularly (500, 1500 and 2500) as shown in Figure 2. The surface was covered with distributed ripples varied in height and depth according to the change of the number of pulses which subsequently increased with increasing the number of pulses. The average surface roughness ranged from 50-280 nm by varying the energy density particularly (15, 20 and 25mJ/cm^2) as revealed in Figure 3. The ripples obtained in this case were disordered, with little number and shallow depth.

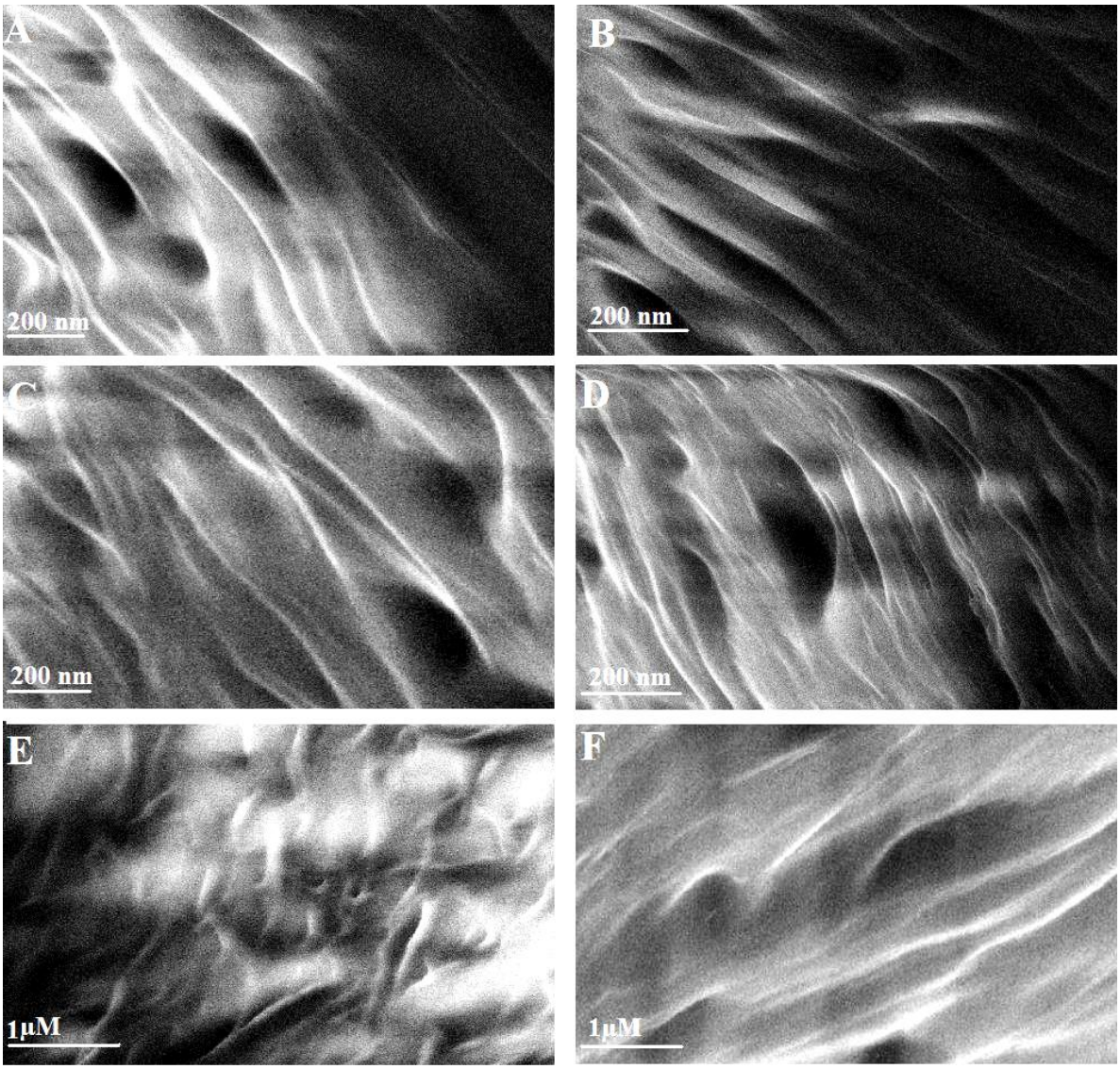

Figure 1 SEM images of modified scaffolds : (A-B) by using pulse rate 5,3 respectively with energy 60 mJ/cm^2, number of pulses 500, (C-D) by using pulse rate 3 Hz with energy 100 mJ/cm^2, number of pulses 500,1500 respectively and (E-F) by using pulse rate 3 Hz, energy 25, 20 respectively mJ/cm^2 and number of pulses 1500.

Figure 4 showed the surface roughness of PCL with varying the pulse rate particularly (2, 3 and 5 Hz). The surface had highly ordered, distributed and consecutive ripples varied in height and depth by changing the pulse rates. The AFM results clearly indicated that the surface roughness has been increased with changing the excimer laser parameters. It should be noted that

the pulse rate had the major effect on the surface roughness. This data was in agreement with the SEM results which subsequently revealed that the laser treated PCL scaffolds provided good adhesive surface for cell attachment and differentiation. These results were in agreement with the data recorded before by Groenendijk, and Meijer 2006 who reported that laser parameters had the ability to change polymers' surface morphology[47].

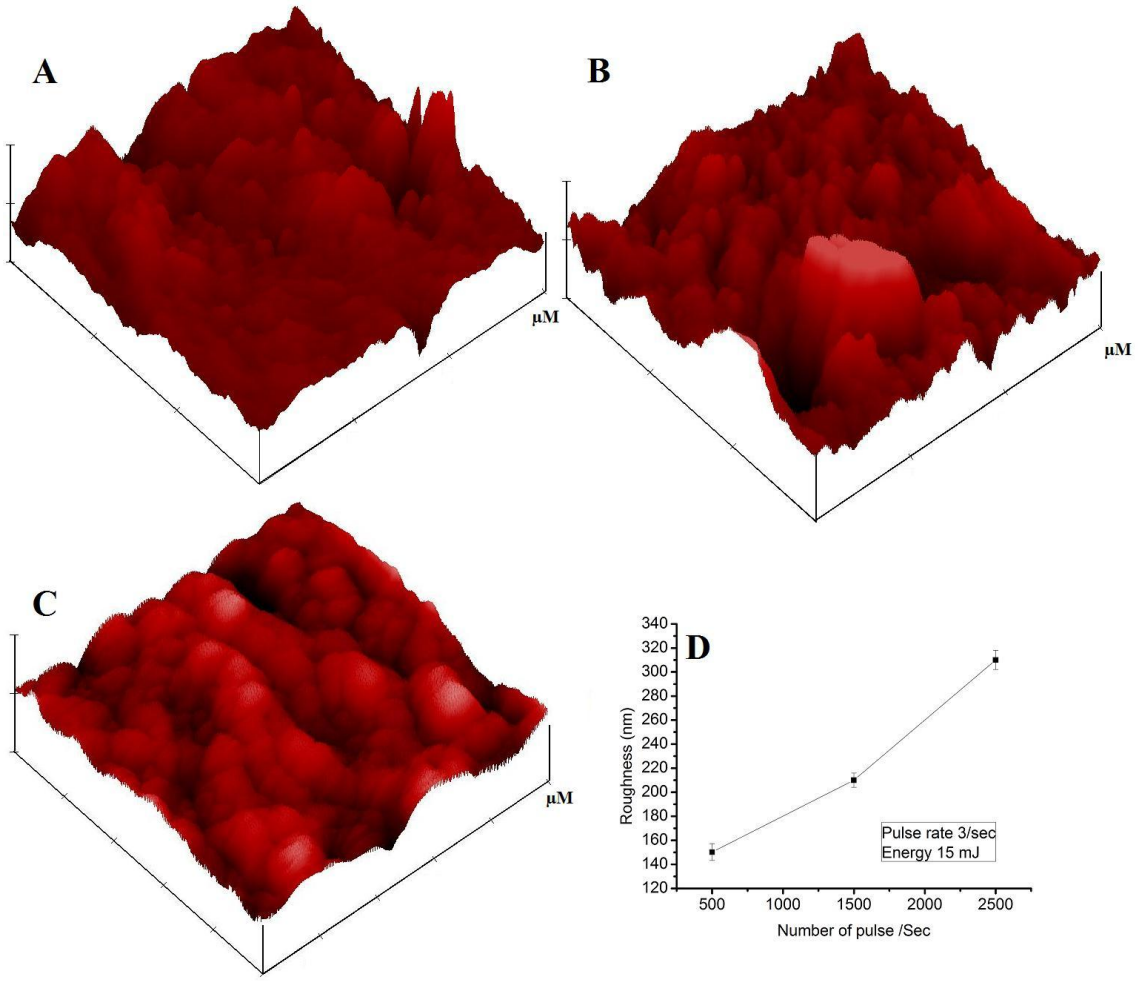

Figure 2 AFM of PCL scaffolds with varied of number of pulses (500, 1500 and 2500) for A, B, and C respectively, energy density 15 mJ/cm^2 and pulse rate 3Hz.

4.3 *In-vitro* degradation test

The scaffold should be biodegradable and bioactive to support the cell differentiation and proliferation. The degradability of the modified and unmodified PCL scaffolds was monitored at 7, 14, 21 and 30 days in culture (Figure 5). Apparently, the degradation rate for both modified and unmodified PCL scaffolds increased gradually up till the day 30. The results indicated that the degradation rate of the modified PCL scaffold was greater than the unmodified scaffold. Laser excitation enhanced the crushability and thermal stability. The presence of nanoripples on the surface of the modified PCL scaffold increased the surface area of the scaffold, hence increasing their degradation. It should be noted that using alkaline medium or higher temperature accelerates the degradation rate of PCL scaffold. In the present work, the degradation rate was measured in SBF to

simulate the *in-vivo* degradation. The results also clearly indicated that, the modified PCL scaffold, have good degradability in *in-vitro* bioactivity, more than the unmodified PCL scaffolds and it will have the ability to make a direct bond to living cells when implanted in the body. These results were in agreement with the results recorded before for degradation rates of PCL [50-51].

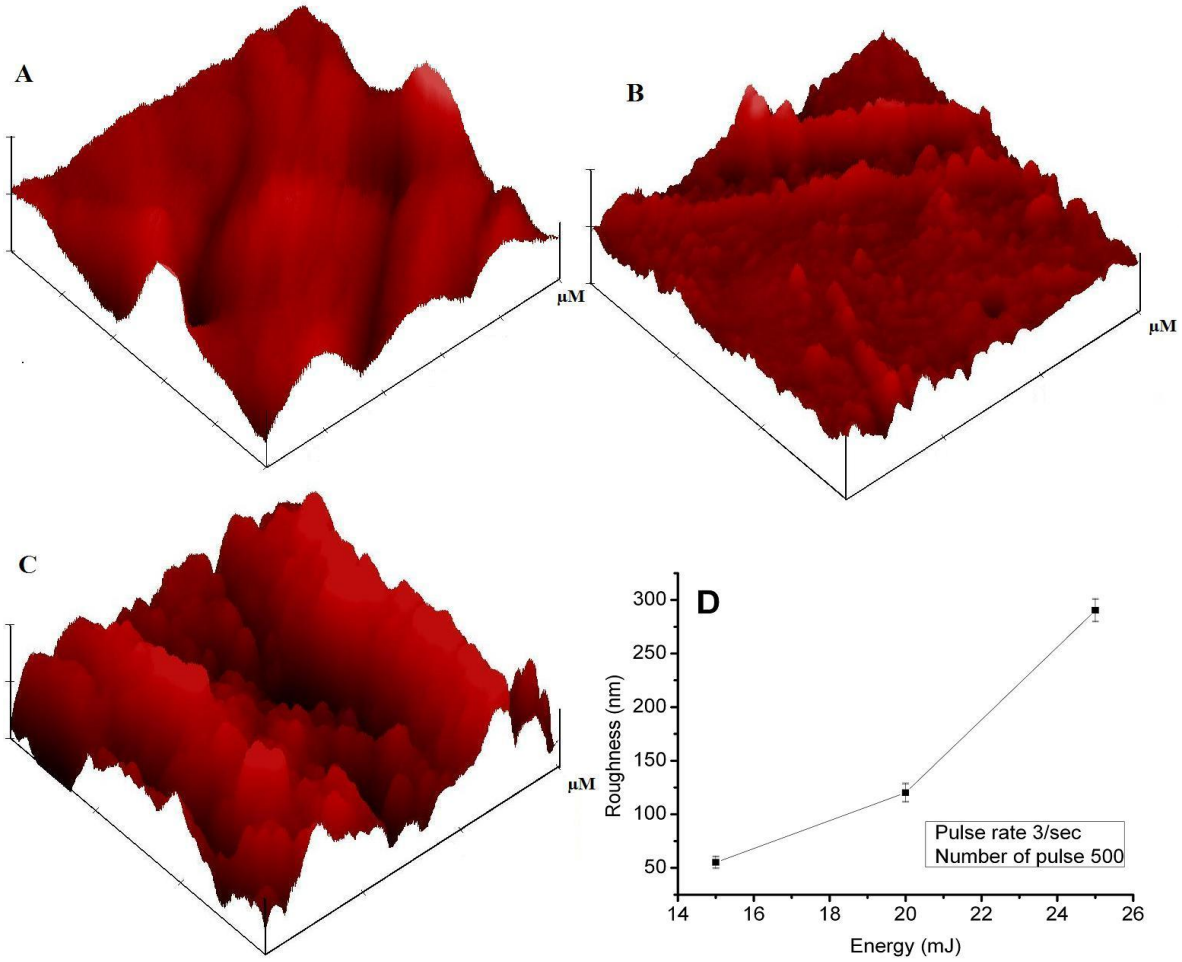

Figure 3 AFM of PCL scaffolds with varied energy density (15, 20 and 25 mJ/cm^2), number of pulses 500 and pulse rate 3Hz

4.4 Cytotoxicity evaluation

The quantitative cell viability on the modified and unmodified PCL scaffolds was evaluated using MTT assay with *human fibroblast cells*. In this assay, only cells that were viable after 24h exposure to the PCL scaffolds were capable of metabolizing the dye efficiently and produce a purple colored precipitate which was analyzed and counted using a micro plate reader as shown in Figure 6. The fibroblasts showed excellent cell viability after 3 weeks of exposure to the modified PCL scaffolds relative to control cells. The number of cells were found to be approximately 3.1×10^6, 5.6×10^6 and 7×10^6 alive cells per well after 1, 2, 3 and 4 weeks of culturing. In contrary, the cells showed poor viability after 3 weeks of culturing on the unmodified PCL scaffold. As the number of cells were 1.9×10^6, 1.5×10^6 and 1.0×10^6 after 1, 2 and 3 weeks of culturing relative to controls. The MTT assay for the cell viability demonstrated that the modified PCL scaffold showed excellent cell viability rather than the unmodified scaffold. The rough surface for the modified PCL scaffold enhanced the cell viability as the cells were able to grow, differentiate and proliferate on the rough

surface for the modified PCL scaffold. In contrary, the cells on the unmodified PCL scaffold undergo detachment due to inadequate and poor adhesion. This data was in agreement with SEM and AFM result.

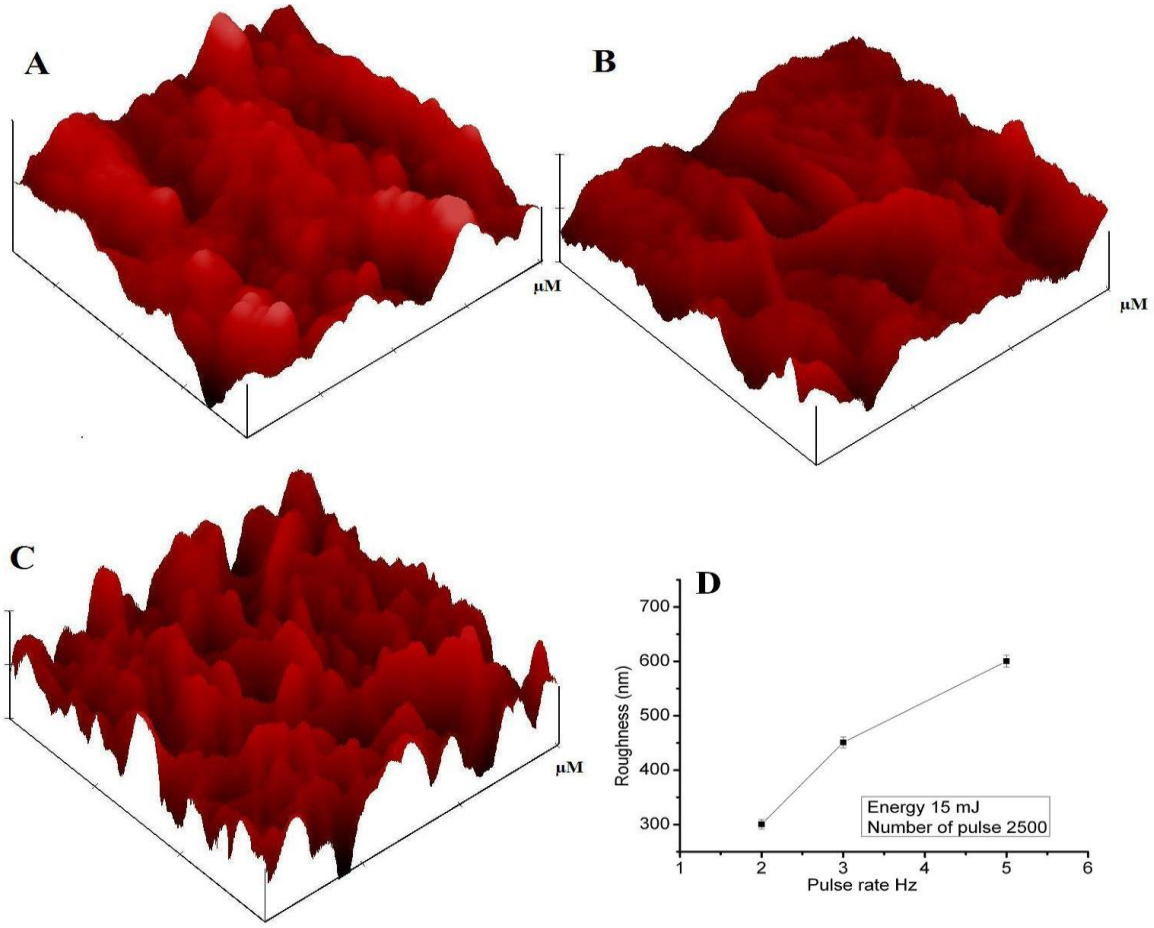

Figure 4 AFM of PCL scaffolds with varied pulse rate (2, 3 and 5Hz), number of pulses 2500 and energy density 15 mJ/cm^2.

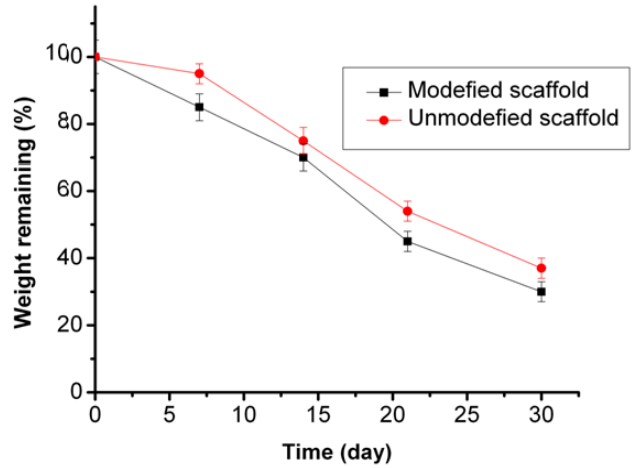

Figure 5 The degradation rate of unmodified and modified PCL scaffold that were prepared by using number of pulses 2500 and energy density 15 mJ/cm^2

4.5 *In-vitro* biological cells imaging

The morphology of cultured fibroblasts on the modified and unmodified PCL scaffolds was observed by SEM as shown in Figure 7. The cells growth and differentiation were monitored after 7, 14 days of culturing for both modified and unmodified PCL scaffolds. The fibroblasts cultured on the modified PCL scaffold have grown faster and proliferated to form elliptical shape after 7 days of culturing Figure 7 (A). After 14 days of culturing the fibroblasts were completely differentiated on the scaffold surface, formed a sheet of cells and matrix like structure Figure 7 (B). In contrary, the fibroblasts cultured on the unmodified PCL scaffolds showed a rod shape with smooth surface after 7 days of culturing Figure 7(C).

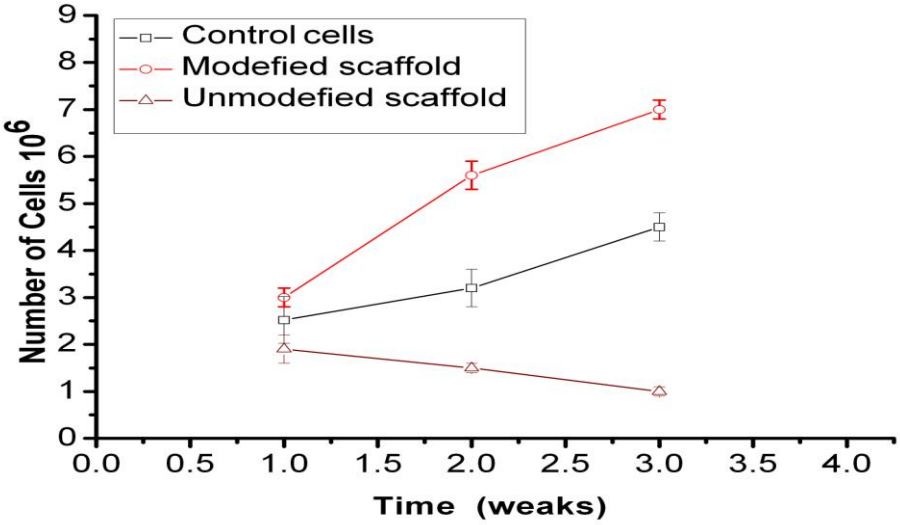

Figure 6 The MTT assay for the cell viability for unmodified and modified PCL scaffolds that were prepared by using number of pulses 2500 and energy density 15 mJ/cm2 relative to control.

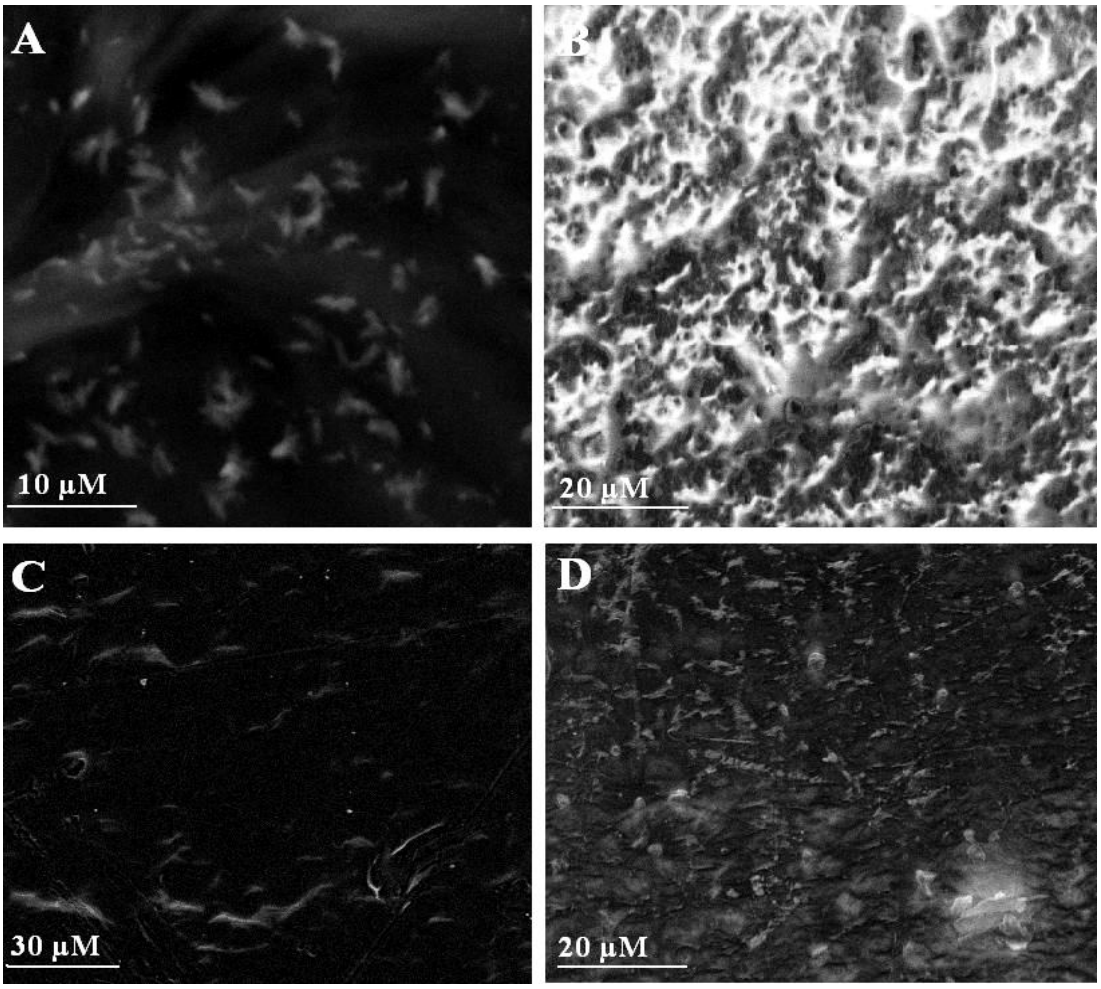

Figure 7 The cell morphology of *human fibroblast cells* on the modified PCL scaffold that were prepared by using number of pulses 2500 and energy density 15 mJ/cm^2 (A) after 7 days of culturing and (B) after 14 days of culturing. The cells morphology on the unmodified PCL scaffold after 7 days of culturing (C) and after 14 days of culturing (D).

Then the cells elongated to longer rods and become spinal in shape with lack of any matrix or sheet of cells Figure 7 (D). This was due to poor attachment of cells on the scaffold surface.

From the results gathered from the cell culture, it was clearly indicated that the laser modified scaffolds were safe material and good carriers for cells as they promoted cell attachment, allowed cell proliferation and differentiation. Therefore, modified scaffolds provide candidate tools for artificial skin and tissue regeneration. These results were in agreement with the data reported before that modified PCL scaffold provided adequate substrate for cell adhesion and differentiation [52-53].

5. Conclusions

In this study, the effect of laser illumination parameters (pulse rate, number of pulses and energy density) on PCL scaffolds surface morphology, roughness, cell viability and cell adhesion were studied. These modified scaffolds could be used for tissue engineering application especially for artificial skin implants. The AFM results demonstrated that the pulse rate had the paramount effect on surface roughness and morphology. The most beneficial surface roughness was obtained with changing the pulse rate and had values ranged from 300 to 600nm. The modified PCL scaffold had good degradability in *in-vitro* bioactivity, more than the unmodified PCL scaffolds reached to 30% of the original weight. From *In-vitro* biological test imaging, the SEM results depicted good adhesion of cells on the modified scaffolds in comparison with the unmodified scaffold. The cells formed complete sheet like structure after 14 days of culturing with lack of any matrix or sheet like structure on the unmodified PCL scaffold. These results clearly indicated that the modified PCL scaffold provided adequate, sufficient surface for cells adhesion, proliferation and could have the ability to make direct bond to living cells when implanted in the body.

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