Formation of Periodic Nanostructures on Medical Polymer with Femtosecond Laser for Control of Cell Spreading

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To enhance the biocompatibility of polymers for medical applications, femtosecond laser was employed to create periodic nanostructures on polymer surfaces, and cell culture tests were conducted on the polymers with periodic nanostructures. Polylactic acid (PLA) and polymethyl methacrylate (PMMA), which are utilized in the fabrication of artificial bones, were utilized. Titanium plate and polymer plate were placed in close contact with each other, and a laser beam was focused on the titanium surface through the polymer to transfer the periodic nanostructures formed on the titanium surface. This process resulted in the formation of nanostructures on PLA and PMMA. Additionally, it was observed that cells exhibited a tendency to stretch along the direction of the periodic nanostructures, in comparison to the polymer that had not undergone laser irradiation. This finding suggests that the nanostructures contributed to an improvement in the biocompatibility of the polymers.

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1. Introduction

Polymers are utilized in the fabrication of artificial ligaments, artificial blood vessels, bone fixation materials, and other biomedical applications. These materials offer lightweight and facile processing properties, rendering them ideal for use in a multitude of biomedical applications [1]. Polylactic acid (PLA) is a polymer with exceptional weather, impact, chemical, oil, and electrical insulation properties. As a biodegradable plastic, PLA is a promising material for clinical applications, including absorption plates, stents, sutures, and bone fixation materials [2]. Polymethyl methacrylate (PMMA) is also anticipated to be utilized in the fabrication of artificial intraocular lenses and artificial bones due to its high visible light transmittance and high impact resistance [3]. However, when these polymers are employed in contact with human tissues (bones, tendons, etc.), it is recognized that the tissue formed on the polymer surface takes time to develop and exhibits strength limitations. Consequently, it is anticipated that surface modification of polymers will result in the development of resin materials with high affinity for human tissues, or in other words, high biocompatibility.

In recent years, research has been conducted to improve biocompatibility by changing the surface properties of materials, such as hydrophilicity, using various surface modification methods [4]. These methods include the formation of microscopic bumps on the surface of hydrophilic materials to create super hydrophobicity [5] and the temporary increase in the wettability of the sample surface with water by applying voltage [6]. However, while these methods can temporarily create super hydrophobicity or superhydrophilicity, they are not permanent. T. Shinonaga et al. reported that by irradiating a titanium substrate with a femtosecond laser, the surface of the substrate was modified with a periodicity of approximately 600 nm [7]. They observed that a specific number of osteoblast cells proliferated along the grooves when osteoblasts were cultured on the periodic microstructure. This result indicates that the biocompatibility of titanium materials can be enhanced by regulating cell growth in a manner that facilitates tissue formation. H. Yashiro et al. created periodic microstructures with a period of approximately 300 nm by irradiating a femtosecond laser beam onto the surface of a square-column material made of zirconium dioxide, which is utilized in artificial joints [8]. The quadrangular prismatic material was then implanted in the tibia of a rabbit, and its adherence strength was measured by a push test. As a result, it was found that the adhesion strength at the interface between the periodic microstructure-formed zirconium dioxide and the bone was improved. This suggests that the formation of periodic microstructures on the surface of biomaterials contributes to the improvement of their biocompatibility with bone. In studies on polymers, femtosecond lasers at 800 nm wavelength were used to texture PMMA [9], collagen, and gelatin [10] surfaces, but no periodic nanostructures were formed. Rebollar et al. used a KrF laser with a wavelength of 248 nm to form periodic nanostructures on polystyrene surfaces to control cell elongation [11], but the molecular structure of the surface was altered due to the use of a shorter wavelength laser. The formation of nanostructures on polymeric materials using direct laser interference patterning (DLIP) and the evaluation of antibacterial properties on their surfaces have also been reported [12], but the biocompatibility has not been evaluated.

In this study, the subject is to form periodic nanostructures on two polymers, PLA and PMMA, using a femto-

second laser, and to clarify the changes in biocompatibility by cell culture tests on the surfaces of polymers with periodic nanostructures. In our previous study, we developed a method to form periodic nanostructures on the surface of PMMA, which has high optical transmittance to the laser wavelength (800 nm), by pressing the polymer onto a Ti substrate and ablating the Ti substrate with a focused laser beam that penetrates the polymer [13]. This method is used to transfer the periodic nanostructure formed on the Ti substrate to the polymer. In this study, we use the same method to form periodic nanostructures on the PLA surface and identify the formation factors involved in the formation of nanostructures on the polymer. In addition, we will conduct cell culture tests by seeding human osteoblasts on the surfaces of PLA and PMMA with periodic nanostructures to clarify the effect of the nanostructures on the direction of cell growth.

2. Experimental conditions

2.1 Femtosecond laser irradiation

The experimental setup and conditions are depicted in Fig. 1 and Table 1, respectively. A Ti:sapphire femtosecond laser (Cyber Laser, IFRIT) with a wavelength of 800 nm, a pulse duration of 150 fs (FWHM), and a repetition rate of 1 kHz was utilized. The experimental setup consisted of three layers: a bottom Ti plate, a middle 1 mm thick polymer plate, and a top quartz glass window. The laser beam, with a diameter of 110 μm at the $1/e^2$ intensity point, was focused to scan the Ti plate using an XY stage. The contact pressure between the Ti plate and the polymer plate was applied by a contact fixture and monitored by a load cell. The reflection of the laser beam at the surface of PLA plate and the absorption within the polymer were less than 10%, and the average laser fluence at the titanium (Ti) surface after transmission through the polymer was set to 0.25 J/cm². A single line was scanned at a length of 5 mm, with a spot size of 110 μm and a scanning speed of 3 mm/s. The pulse overlap number was 36.7 pulses at the scanning speed of 3 mm/s.

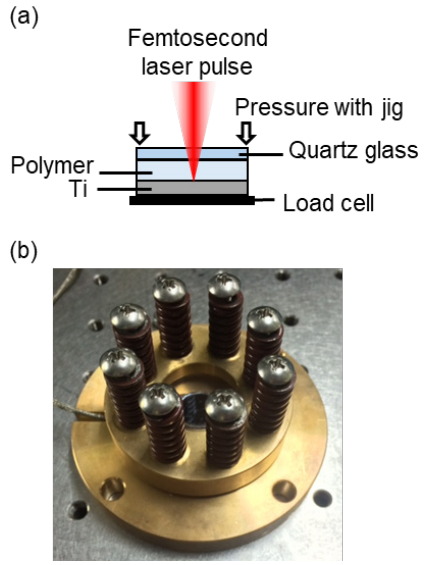

Fig. 1 (a)Schematic diagram of laser irradiation-method and (b) photo of the jig.

The periodic nanostructures on the PLA plate were observed with a scanning electron microscope (SEM; Keyence, VE-9800) and an atomic force microscope (AFM; SII Nanotechnology, AFM5100N). The period and depth were defined by the average value of 200 points measured from valley to valley of the periodic nanostructures. Energy dispersive X-ray spectrometry (EDX; Oxford Instruments, X-Act) was employed to analyze the elements on the PLA surface before and after laser irradiation.

Table 1 Experimental conditions

Femtosecond laser	Ti:Sapphire
Wavelength	800 nm
Pulse width	150 fs
Repetition rate	1 kHz
Spot size	110 μm
Laser fluence	0.25 J/cm2
Scanning speed	3.0 mm/sec
Hatching distance	100 μm
Ti pressure	0 ,600, 1200, 1800, 2400 kPa

2.2 Cell cultivation test

To assess the relationship between the periodic nanostructures on the polymer plate and cell spreading, cell culture tests were conducted using osteoblasts (MG-63) on the polymer plate with and without periodic nanostructures, as illustrated in Fig. 2(a). Both samples and cells were incubated in 5% CO_2 at 37°C for 4.5 hours [14]. Following rinsing with phosphate-buffered saline (PBS) and fixation in 8% paraformaldehyde (PFA), the cells were immunostained and observed with a fluorescence microscope (BZ-9000, Keyence Co.) to examine the cell spreading direction. The nucleus, actin, and focal adhesion of the cells on Ti appeared blue, red, and green, respectively.

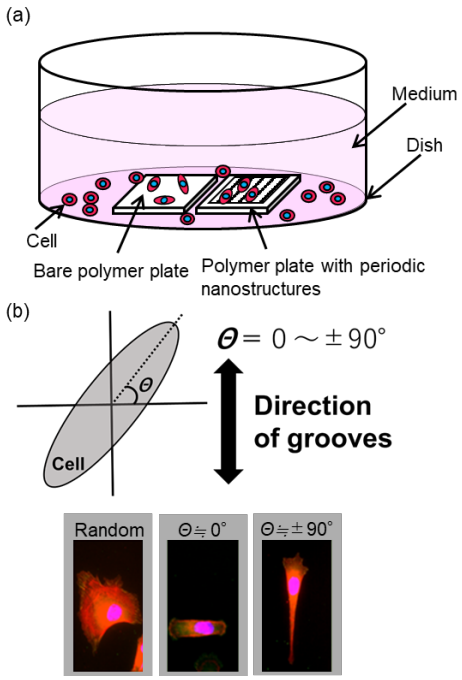

Fig. 2 (a) Schematic diagram of cell cultivation test and (b) method for the determination of direction of cell spreading with orientation angle.

As illustrated in Fig. 2(b), the angle between the direction of cell spreading and a horizontal line is assumed to be the orientation angle θ. The periodic nanostructure was formed at an angle of θ = 90°, and the cell spreading frequencies were evaluated after the cell culture test by counting the number of cell orientations three times. The cell spreading frequency was calculated as $A = 100 \times N_\theta / N_{all}$, where A (%) is the percentage of cell spreading frequency, and N_θ / N_{all} are the number of cell orientations at θ = 0 °, ±15 °, ±30 °, ±45 °, ±60 °, ±70 °, and 90 °, respectively.

3. Experimental results and discussion

3.1 Formation of periodic nanostructures on PLA

Figure 3a (e) depicts the SEM (AFM) images of a non-irradiated PLA. Subsequent to this, non-irradiated unstructured surfaces will be referred to as bare surfaces. The bare surface of the PLA exhibited a smooth appearance and did not exhibit periodic structures. Figure 3b (f) shows the SEM (AFM) images of the laser-irradiated PLA at a pressure of 0 kPa. No periodic nanostructures were formed on the PLA plate. Figure 3c, d (g, h) shows the SEM (AFM) images of the laser-irradiated PLA at a pressure of 600 kPa and 1800 kPa, respectively, at a scanning speed of 3.0 mm/s and a laser fluence of 0.25 J/cm².

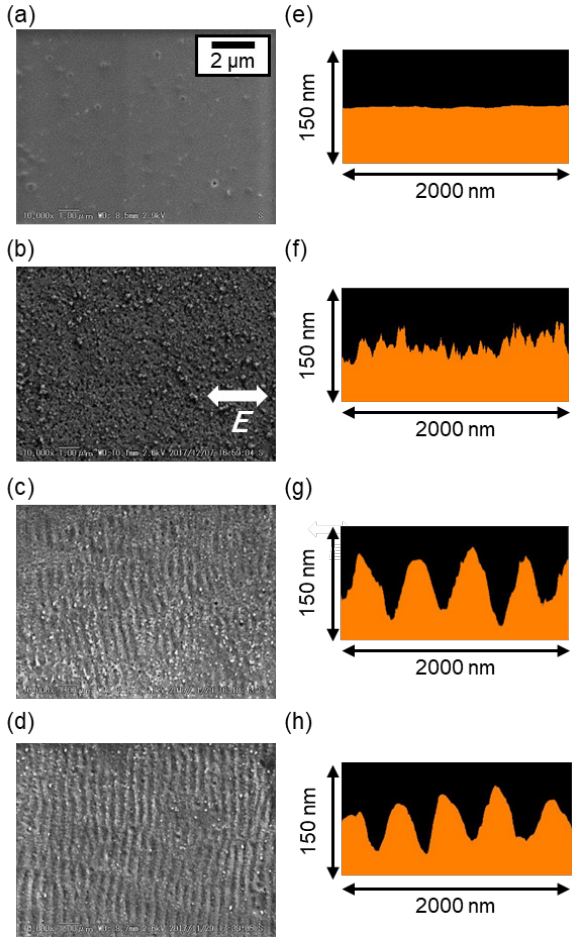

Fig. 3 a, b, c, and d: SEM images of the bare PLA surface and laser irradiated PLA surface at the pressure of 0 kPa, 600 kPa, and 1800 kPa, respectively. e, f, g, and h: AFM images of the bare PLA surface and laser irradiated PLA surface at the pressure of 0 kPa, 600 kPa, and 1800 kPa, respectively.

Figure 4 illustrates the impact of pressure on the period and depth of the nanostructure. The pressure values examined were 600, 1200, 1800, and 2400 kPa, with a laser fluence of 0.25 J/cm² and a scanning speed of 3.0 mm/s. Consequently, the minimum contact pressure between PLA and Ti to form the periodic nanostructure is 600 kPa. The period and depth of the periodic nanostructures are constant at approximately 430 nm and 65 nm, respectively. The average depth value decreases with increasing pressure, but the extent of this change is within the range of the error bars, indicating that pressure does not exert a significant influence on the observed phenomenon. The period of the nanostructures formed on the Ti plate is almost identical to the period of the nanostructures formed on the PLA plate [15]. The structures are oriented perpendicular to the electric field polarization vector of the laser. This is consistent with previous studies [16]. EDX analysis revealed that Ti was not detected in the non-irradiated area. However, Ti with a mass fraction of 0.2% was observed in the irradiated area. Ultrasonic cleaning was found to remove Ti from the PLA surface. Based on these observations, it can be proposed that irradiating the Ti plate with a femtosecond laser generates ablated plumes, which produce nanostructures on the Ti plate. During this process, the Ti plumes are ejected from the Ti plate and subsequently etch the PLA surface, which replicates the nanostructures formed on the Ti plate. These results indicate that the ablated Ti plumes are responsible for the formation of the periodic nanostructures on the PLA surface.

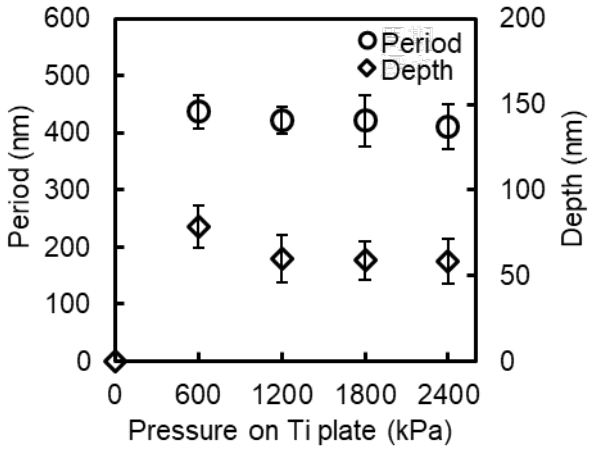

Fig. 4 Relationship between the period of periodic nanostructures on PLA plate formed with femtosecond laser and the pressure on Ti.

The periodic nanostructures formed on the Ti surface in air had a period of approximately 600 nm, whereas the period of the periodic nanostructures formed on the PLA and Ti surface changed to approximately 410 nm when the PLA was adhered to the Ti surface. The surface plasmon polariton (SPP) is postulated as the formation mechanism of the periodic nanostructures. The change in the dielectric constant of the Ti surface from 1 (in air) to 2.3 (PLA) is considered to have caused the period of the SPP standing wave to decrease, resulting in a smaller periodic nanostructure. The relationship between the periodic nanostructures formed on titanium substrates and the surface dielectric

constant has been discussed in a previous study[15], and the same tendency was observed for the periodic nanostructures transferred to the polymer surface. When the pressing pressure was 0 kPa, no periodic nanostructures were formed on the PLA surface. In contrast, periodic nanostructures were formed on the Ti substrate surface with the same periodicity as when the PLA and Ti were irradiated in air. The macroscopic changes in surface morphology due to laser irradiation were quantified using AFM, revealing that the PLA was scraped off by approximately 200 nm and the Ti substrate was swollen by 100 nm after laser irradiation. The underlying cause of the swelling of the Ti surface due to laser irradiation requires further investigation. It is hypothesized that the surface may have become porous due to redeposition of ablated Ti

3.2 Cell spreading direction

To assess cellular behavior on PLA surfaces with periodic nanostructures, culture tests were conducted using human osteoblasts. The laser irradiation conditions were as follows: fluence of 0.25 J/cm², scanning speed of 3 mm/s, hatching distance of 100 µm, and pressure of 1800 kPa. The center of a 10 mm x 10 mm PLA plate was irradiated to form periodic nanostructures with a 5 mm x 5 mm area. For comparison, the same irradiation conditions were employed to form periodic nanostructures on PMMA, which is utilized as the same medical polymer. Cell tests were conducted to assess the impact of these structures on cellular viability. The results demonstrated that periodic nanostructures with a period of 430 nm and a depth of 65 nm were formed on the PLA surface, while periodic structures with a period of 410 nm and a depth of 70 nm were formed on the PMMA surface.

Figure 5 presents fluorescence microscope images of bare (a) PLA and (b) PMMA plates and laser-irradiated (c) PLA and (d) PMMA after 4.5 hours of the cell cultivation test. The cells exhibited a tendency to spread freely and randomly on the bare PLA and PMMA surface. The cell spreading observed on the PLA and PMMA plates with periodic nanostructures occurred in the direction of the grooves. The orientation angles of the cells on both surfaces were quantified and measured using a fluorescence microscope. Figure 6 a (b) illustrates the percentage of cells as a function of the angle of cell spreading on PLA (PMMA). The percentage of cells oriented on the bare polymer surface and the laser irradiated polymer surface was shown with white bar chart and bar chart of the diagonal line of the right shoulder, respectively. On the bare surfaces of both PLA and PMMA, the majority of cells exhibited an elongated morphology in random directions. Furthermore, even those cells that were elongated in a single direction exhibited considerable variability in their direction of elongation. Conversely, on the surfaces of PLA and PMMA with periodic nanostructures, the percentage of cells extending in random directions decreased, and the percentage of cells following the direction of the periodic nanostructures exceeded the majority. This trend was found to be independent of the type of polymer and was not affected by differences in the periodic nanostructures. This phenomenon was found to be consistent with the observations reported by Shinonaga et al. [7] and Matsugaki et al. [17], who had previously investigated the cell spreading behaviour along nanostructures on Ti and TiO_2 surfaces.

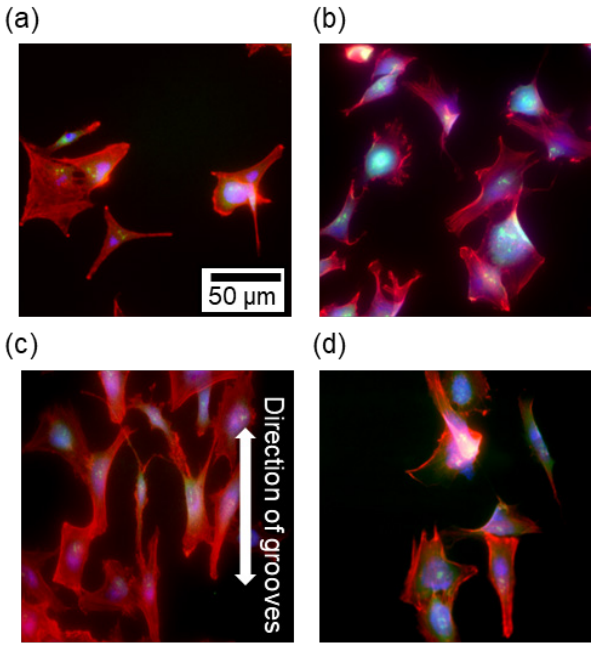

Fig. 5 Fluorescence microscope images of after cell cultivation on bare (a) PLA and (b) PMMA surface and (c) PLA (d) PMMA with periodic nanostructures. The directional grooves of the periodic nanostructures were indicated by the arrow.

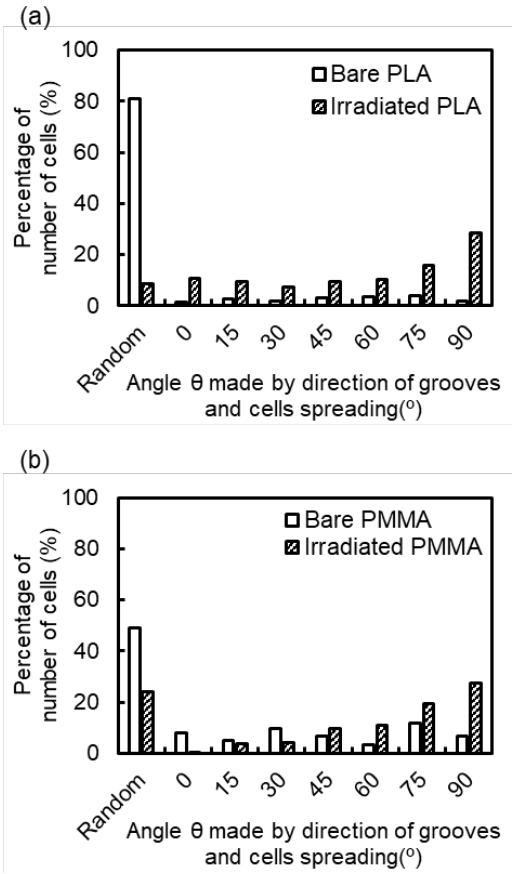

Fig. 6 The percentage of number of cells as a function of angle of cell spreading on (a) PLA and (b) PMMA plate.

These findings indicate that periodic nanostructures formed on the resin surface are an effective means of controlling the direction of cell growth, and that a 20 nm difference in period does not affect the control of cell growth. Only approximately half of the seeded cells extended in the direction of the periodic nanostructures. Future research will be directed towards the investigation of the shape of the periodic nanostructures that can control a larger number of cells, as well as the search for a method to create such structures.

4. Conclusion

Periodic nanostructures were formed by focused femtosecond laser pulses irradiation of PMMA and PLA, a type of medical polymer. It was found that the direction of cell extension was controlled on the surface of the nanostructured surfaces, thereby improving biocompatibility. Despite the challenges associated with directly forming nanostructures on polymers, periodic nanostructures were generated on the surface of PLA through a process involving the close contact of the polymer and Ti, followed by the focused irradiation of a laser beam through the polymer to transfer the nanostructures formed on the Ti substrate surface onto the polymer. It was demonstrated that approximately half of the cells on the surfaces of PLA and PMMA with periodic nanostructures exhibited the capacity to regulate the direction of cell growth. Furthermore, the nanostructures formed on the polymers contributed to the enhancement of the biocompatibility of the polymers.

Acknowledgments

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