

ORIGINAL ARTICLE

Enhanced cellular affinity for poly(lactic acid) surfaces modified with titanium oxide

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A surface modification method of biocompatible poly(lactic acid) (PLA) with titanium oxide (TiO_x) is proposed. Tetraethoxytitanium(IV) (TEOT) was used to construct a TiO_x network with a few residual ethyl groups via an acid-catalyzed sol–gel reaction exploiting surface segregation. The surface of the resulting PLA–TiO_x composite films was flat and homogeneous when the TEOT loading was < 5 wt%. The properties of the PLA–TiO_x composite films were examined by microscopy to explore the fibroblast affinity for the scaffold surface. The number of cells attached to the PLA–TiO_x composite films increased with increasing surface TiO_x content. Cell morphology was also dependent upon the surface TiO_x content, and the attached cells on the composite film were better spread than those on the pristine PLA film. These results clearly show that our proposed method is a useful technique to improve the affinity of cells for PLA surfaces.

Polymer Journal (2015) 47, 505–512; doi:10.1038/pj.2015.30; published online 13 May 2015

INTRODUCTION

How to regulate the interaction between cells and material surfaces is one of the key fundamental topics in biotechnology and biomedical fields.^{1–3} Numerous types of cellular scaffolds have been developed using organic^{4–7} and inorganic^{8–10} polymeric materials, and their composites^{11,12} via various chemical and physical processing methods. In addition, how and why polymer properties affect the cell behavior, such as adhesion, migration, proliferation and differentiation, have been extensively studied. Until now, it has been accepted that the surface parameters of polymers, such as chemical composition,² surface-free energy,^{13,14} topology,^{5,7,15} roughness,^{16,17} crystallinity^{16–18} and so on, have an important role in controlling the cell function in *in vitro* culturing systems.

Biocompatible polymers such as poly(lactic acid) (PLA), poly(glycolic acid) and their copolymers have attracted a great deal of attention as scaffold materials for cell culturing due to their high biocompatibility.^{17–20} However, further application of these polymers without modification will be challenging because of inadequate cell–surface interactions.^{13,21–24} For example, a low affinity of the scaffold surface with the cells will be problematic, leading to an inflammatory response after implantation. To improve the affinity of the PLA-made scaffold with the cells, the surface was modified by incorporating various functional groups using a variety of approaches, such as copolymerization, graft polymerization^{25–31} and plasma irradiation.^{32–35} After such modifications were performed, the surface properties including the hydrophilicity and chemical reactivity were altered, and the cell functionality was enhanced on the polymer scaffold surfaces. However, despite the abundance of literature documenting the surface modification of PLA, it appears that their

industrial use remains limited. This is a consequence of the complicated and time-consuming methods currently used for the surface modification of PLA. In addition, it is quite difficult to introduce functional groups to an all-purpose polymer without damaging the material. Thus, a more versatile method that can be applied to such polymers is desirable.

Recently, we applied the sol–gel reaction of tetraethoxysilane (TEOS) to modify the surfaces of polyisoprene³⁶ and polystyrene³⁷ films. TEOS is fed into a polymer solution and reacted to form an inorganic network before and/or during the film solidification and is then segregated at the film surface. This confers hardness and antioxidant properties to the surface. In this study, we extend our simple method to modify the surface of a PLA scaffold with tetraethoxytitanium(IV) (TEOT) rather than TEOS, which forms a titanium oxide (TiO_x) network after the sol–gel process. TiO_x is a well-known biocompatible inorganic material due to its corrosion resistance, mechanical properties, inertness and so forth, when in contact with biocomponents.^{38–43} TiO_x has been used in clinical applications as implants, specifically as a bone replacement. Here thin films of PLA–TiO_x composite are prepared and used as a scaffold for cell culture. We clearly demonstrate that the small amount of TiO_x network formed at the scaffold surface influences the cell behavior, including cell adhesion and proliferation.

EXPERIMENTAL PROCEDURE

Preparation of PLA–TiO_x composite films

PLA prepared via ring opening polymerization of racemic DL-lactide was purchased from Polymer Source (Montreal, Quebec, Canada) and purified by reprecipitation. Using gel permeation chromatography analysis, the

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Received 4 January 2015; revised 17 March 2015; accepted 17 March 2015; published online 13 May 2015

number-average molecular weight (M_n) and polydispersity index were determined to be 92k and 1.93, respectively. A sol was prepared by acid-catalyzed hydrolysis of TEOT (Sigma-Aldrich, St Louis, MO, USA). TEOT mixed with ethanol, ultrapure water and hydrochloric acid at the desired ratio, called TEOT_{hyd}, was stirred at room temperature under a nitrogen atmosphere for 10 min. The rate of hydrolysis of TEOT was regulated to prevent complete gelation. TEOT_{hyd} was then added to a solution of PLA in tetrahydrofuran, and it was stirred for 30 min to obtain a homogeneous solution. PLA thin films with a thickness of ~170 nm that contained titanium oxide (PLA-TiO_x composite) were prepared by spin-coating the solutions onto silicon wafers and borosilicate cover glasses. The films were annealed at 353 K under reduced pressure for 24 h. The loading ratios of PLA/TEOT (weight/weight) selected were 97/3, 95/5, 90/10 and 80/20. As a reference, homogeneous PLA and TEOT_{hyd} films were also prepared and labeled 100/0 and 0/100 PLA/TEOT (weight/weight).

Surface characterization

The surface morphology of the films was characterized by atomic force microscopy (E-sweep with a SPI3800 controller, Hitachi High-Technologies, Tokyo, Japan) using dynamic force mode at room temperature. A cantilever tip with a nominal spring constant of 1.3 N m^{-1} and a resonance frequency of 27 kHz was used for the observations. Static and dynamic contact angle measurements were performed using a Drop Master 500 (Kyowa Interface Science, Niiza, Japan). A droplet of pure water was used as a probe liquid. For dynamic contact angle measurements, a film on a silicon wafer was tilted until a 20- μl water droplet began to slide down the surface. The advancing (θ_a) and receding (θ_r) angles were measured and used to calculate the contact angle hysteresis ($\Delta\theta = \theta_a - \theta_r$). The surface chemical composition of the PLA-TiO_x composite films was examined using X-ray photoelectron spectroscopy (XPS, PHI 5800 ESCA system, Physical Electronics, Chanhassen, MN, USA) with a monochromatized AlK α source. The emission angle was fixed at 45°, corresponding to an analytical depth of ~7 nm from the outermost surface. The C_{1s} peak was calibrated using the binding energy of neutral carbon (285.0 eV) to correct for charging energy shifts.

Cell culturing

The PLA-TiO_x composite films on the borosilicate substrates were used as a scaffold for cell culturing. The scaffold films were washed with a phosphate-buffered saline solution, and, then, they were placed on the base of a 24-well culture dish filled with commercially available culture media, normal Roswell Park Memorial Institute 1640 (Life Technologies, Tokyo, Japan), with or without 10% fetal bovine serum. Suspensions of fibroblast L929 (RIKEN BRC Cell Bank, Tsukuba, Japan), derived from mice, were seeded onto the scaffold films at 5.0×10^4 cells per well. The cultures were maintained at 310 K (37 °C) in a humid atmosphere containing 5% CO₂. The cell adhesion state was evaluated using phase contrast microscopic observations. To assess the cell viability accurately, fluorescence microscopy was used after cell staining with a

LIVE/DEAD solution (Life Technologies) containing calcein O₃O₃-diacetate tetrakis(acetoxymethyl) ester (Calcein-AM, excitation/emission maxima ($\lambda_{ex}/\lambda_{em}$) of 494/517 nm) and ethidium homodimer-1 (EthD-1, $\lambda_{ex}/\lambda_{em}$ of 528/617 nm).⁴⁴ Observations were performed using a GFP-BP filter cube (center wavelength/bandwidth (CWL/BW) = 470/40 nm for excitation, CWL/BW = 535/50 nm for emission, Keyence, Osaka, Japan) and a TRITC filter cube (CWL/BW = 540/25 (nm/nm) for excitation, CWL/BW = 605/55 nm/nm for emission, Keyence), respectively.

Furthermore, fluorescence microscopy was used to examine the formation of stress fibers in the cells adhered onto the polymer scaffolds. The cells were immobilized onto the scaffolds before the observations. After the removal of the culture medium, the samples were fixed with 4% paraformaldehyde for 30 min and washed with phosphate-buffered saline at 310 K three times. Alexa Fluor 594 phalloidin ($\lambda_{ex}/\lambda_{em}$ of 581/609 nm, Life Technologies) was used to stain the F-actin filament formed in the fibroblast. All cell nuclei were stained with 4',6-diamidino-2-phenylindole ($\lambda_{ex}/\lambda_{em}$ of 358/461 nm for bound to double-stranded DNA, Life Technologies).

RESULTS AND DISCUSSION

Surface properties of PLA-TiO_x composite films

Figures 1a–c show the topographic images for PLA and the PLA-TiO_x composite films. Figures 1d–f show the relative height profiles along lines in the corresponding topographic images. The surface of the PLA-TiO_x composite films with a loading weight ratio of 97/3 and 95/5 was quite flat and featureless. The root mean square roughness values for these films were 0.87 and 1.4 nm, respectively. These results are almost comparable to the PLA homogeneous film, which exhibited a value of 0.67 nm. When the weight ratio of TEOT exceeded 10%, the surface roughness increased significantly (data not shown). The root mean square roughness for the 90/10 and 80/20 films were 16 and 34 nm, respectively. In the following section, only 97/3 and 95/5 films are discussed.

Table 1 summarizes the wettability of the PLA and PLA-TiO_x composite films. Although the static contact angle of water (ϕ_{water}) on the PLA film was (78.8 ± 0.4)°, for the 97/3 and 95/5 PLA-TiO_x composite films the static contact angles were (83.0 ± 0.3)° and (84.5 ± 0.5)°, respectively. These results are contrary to what was expected on the addition of hydrophilic TiO_x. A possible explanation for this result may be that the TEOT hydrolysis reaction did not proceed to completion. Therefore, some ethyl groups originating from the unhydrolyzed TEOT remain at the surface of the composite film, reducing its hydrophilicity compared with TiO_x. To confirm this hypothesis, XPS measurements were performed. Figures 2a and b

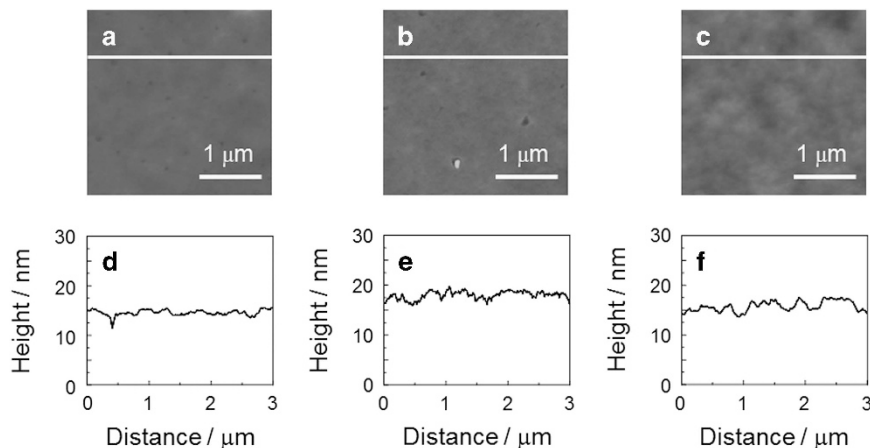


Figure 1 AFM topographic images for (a) homogeneous PLA film, (b) 97/3 (wt/wt) PLA-TiO_x composite film and (c) 95/5 (wt/wt) PLA-TiO_x composite film. Sectional views (d–f) along lines in (a–c). AFM, atomic force microscopy; PLA, poly(lactic acid); TiO_x, titanium oxide.

Table 1 The wettability of the PLA and PLA-TiO_x films evaluated by static and dynamic contact angle measurements

Sample	PLA/TEOT ratio (wt/wt)	Static contact angle (degree)		Dynamic contact angle (degree)	
		ϕ_{water}	θ_a	θ_r	$\Delta\theta$
PLA	100/0	78.8 ± 0.4	94.9 ± 1.2	60.0 ± 0.9	34.9
PLA-TiO _x composite	97/3	83.0 ± 0.3	96.2 ± 0.8	66.3 ± 1.1	29.9
PLA-TiO _x composite	95/5	84.5 ± 0.5	95.2 ± 1.5	64.0 ± 2.3	31.2

Abbreviations: PLA, poly(lactic acid); TEOT, tetraethoxytitanium(IV); TiO_x, titanium oxide. Data represent the mean ± s.d. (*n* = 10).

show the survey spectra for the homogeneous PLA film and the 95/5 PLA-TiO_x composite films, respectively. For the composite film, a weak peak assigned to Ti_{2p} was observed at ~460 eV in addition to the intense C_{1s} and O_{1s} peaks at ~285 and 540 eV, respectively. Figures 2c and d show the C_{1s} core-level spectra for the PLA-TiO_x composite and TEOT_{hyd} homogeneous films, respectively. In the case of the PLA-TiO_x composite film, the C_{1s} peaks corresponding to neutral, ether and carbonyl carbons were observed at 285.0, 286.5 and 289.0 eV, respectively (Figure 2c). Because PLA chains contain these three carbons types, the spectrum obtained is reasonable. Peaks associated with neutral and ether carbons were visible in the TEOT_{hyd} film C_{1s} spectrum (Figure 2d). This result indicates that the hydrolysis of TEOT was incomplete under the current conditions. Taking into account the integrated intensity of the C_{1s} and Ti_{2p} spectra,⁴⁵ the

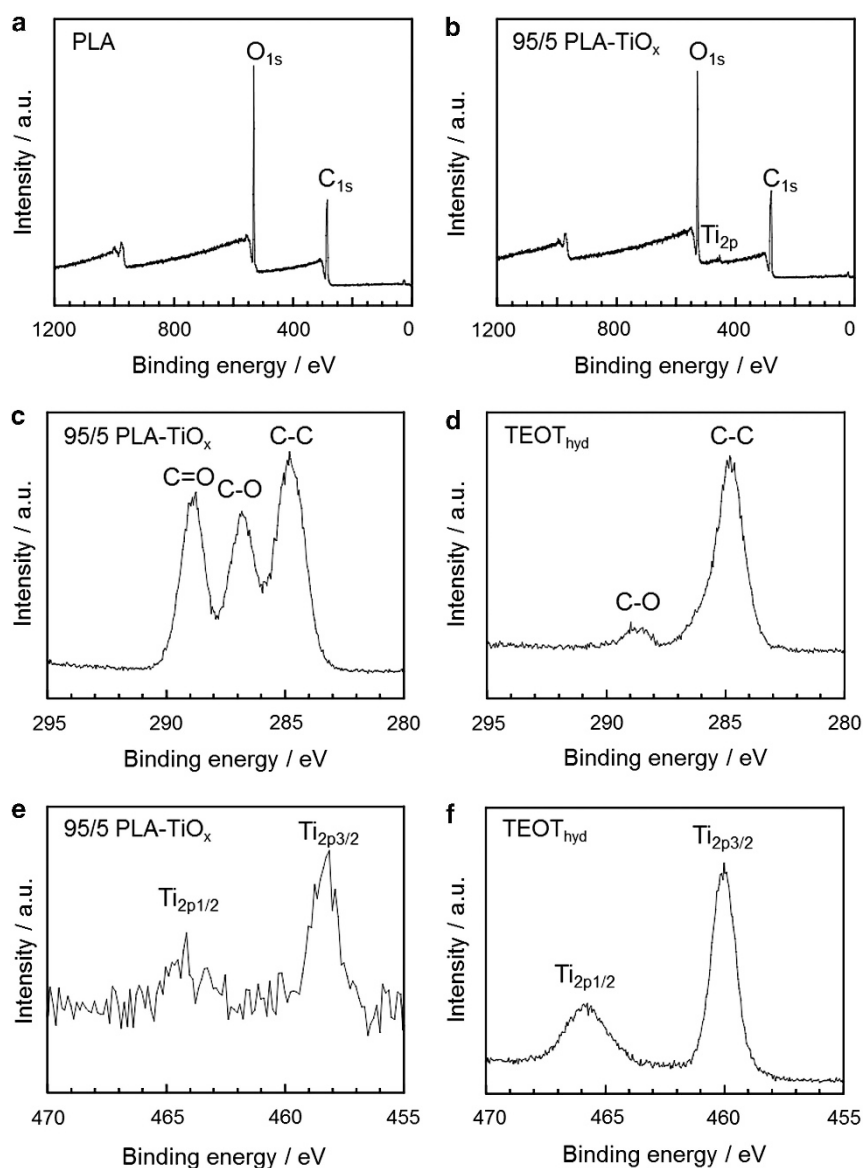


Figure 2 XPS survey spectra for a (a) homogeneous PLA film and (b) 95/5 (wt/wt) PLA-TiO_x composite film. XPS core-level spectra of C_{1s} for a (c) 95/5 PLA-TiO_x composite film and (d) TEOT_{hyd} homogeneous film. The neutral C_{1s} peak was assigned to a binding energy of 285.0 eV to correct for the charging energy shift. XPS core-level spectra of Ti_{2p} for a (e) 95/5 PLA-TiO_x composite film and (f) TEOT_{hyd} film. PLA, poly(lactic acid); TEOT, tetraethoxytitanium(IV); TiO_x, titanium oxide; XPS, X-ray photoelectron spectroscopy.

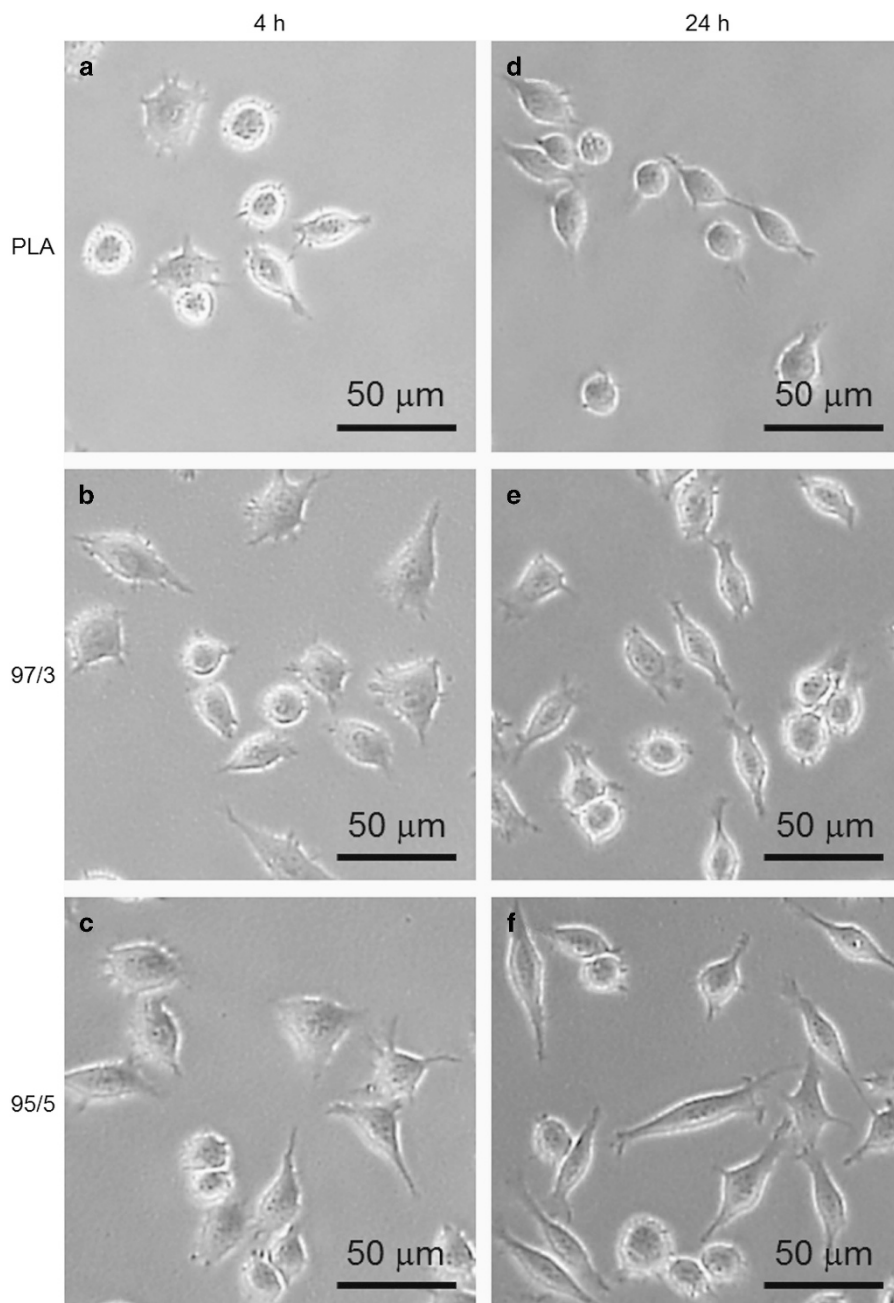


Figure 3 Phase contrast images of L929 fibroblasts adhered on (a, d) a homogenous PLA film, (b, e) 97/3 (wt/wt) PLA-TiO_x composite film and (c, f) 95/5 (wt/wt) PLA-TiO_x composite film after culturing under serum-free conditions for (a–c) 4 and (d–f) 24 h. Scale bars correspond to 50 μm. PLA, poly(lactic acid); TiO_x, titanium oxide.

amount of TEOT that was hydrolyzed was determined to be ~40%. Thus, although the difference in the C_{1s} XPS spectra between the homogeneous PLA film and the PLA-TiO_x composite film was very small, the C_{1s} peaks shown in Figure 2c may represent the summation of the peaks derived from PLA chains and the hydrophobic ethyl groups originating from TEOT that remained after hydrolysis. This is in good agreement with the higher ϕ_{water} observed for the PLA-TiO_x composite film compared with the pristine one.

Figures 2e and f show the Ti_{2p} core-level spectra obtained for the PLA-TiO_x composite and TEOT_{hyd} homogeneous films, respectively. Two peaks corresponding to different spin orbit components, (Ti_{2p} 3/2) and (Ti_{2p} 1/2), were observed. It is known that Ti_{2p} peaks

from the pure metal (Ti) are observed at 454.6 and 460.6 eV, respectively. However, their binding energies for the PLA-TiO_x composite film were shifted by ~3.5 eV to 458.3 and 464.0 eV, respectively. This indicates that the Ti was oxidized in the composite film to Ti⁴⁺.^{46,47} That is, TiO_x exists at the surface of the PLA-TiO_x composite film. The surface Ti content at a depth of 6.9 nm was calculated to be 2.2 wt% from the ratio of the integrated intensity of C_{1s} and Ti_{2p} spectra.⁴⁷ The Ti content in the 95/5 PLA-TiO_x composite film was calculated to be 1.1 wt%, assuming the TEOT is 40% hydrolyzed. Therefore, the partially hydrolyzed TEOT formed Ti-O-Ti networks at the film surface. Unfortunately, in the case of the 97/3 PLA-TiO_x composite film, the Ti_{2p} peaks were not clearly

observed. However, the ϕ_{water} for this film was also larger than that for the homogeneous PLA film. This indicates the 97/3 PLA-TiO_x

composite film also has the Ti-O-Ti network with ethyl groups at the surface similarly to the 95/5 PLA-TiO_x composite film.

Regarding the dynamic contact angle for the prepared films, it was noted that the θ_a values were almost constant regardless of the fractional amount of TiO_x; however, the $\Delta\theta$ values for the PLA-TiO_x films were smaller than that for the homogeneous PLA film by $\sim 4^\circ$. This is due to the larger θ_r values for the composite films, as shown in

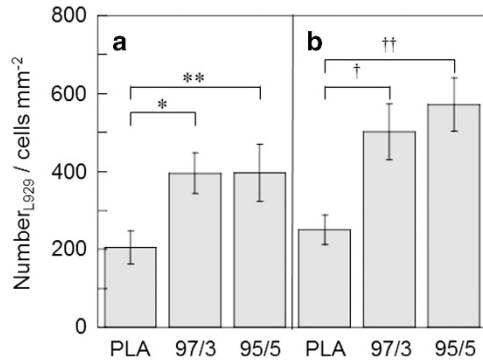


Figure 4 The number of cells adhered on the PLA and PLA-TiO_x (97/3 and 95/5) composite films cultured under serum-free conditions for (a) 4 and (b) 24 h. The data represent the mean ± s.d. * $P < 0.005$; ** $P < 0.005$; † $P < 0.005$; †† $P < 0.005$ (Student's *t*-test). PLA, poly(lactic acid); TiO_x, titanium oxide.

Table 2 Peak position and full width at half maximum (FWHM) of the normal distribution curves in Figure 5

Sample	PLA/TEOT ratio (wt/wt)	Culturing time (h)	Peak position (μm^2)	FWHM (μm^2)
PLA	100/0	4	582	444
PLA-TiO _x composite	97/3	4	784	810
PLA-TiO _x composite	95/5	4	796	687
PLA-TiO _x composite	100/0	24	536	416
PLA-TiO _x composite	97/3	24	774	625
PLA-TiO _x composite	95/5	24	968	855

Abbreviations: PLA, poly(lactic acid); TEOT, tetraethoxytitanium(IV); TiO_x, titanium oxide.

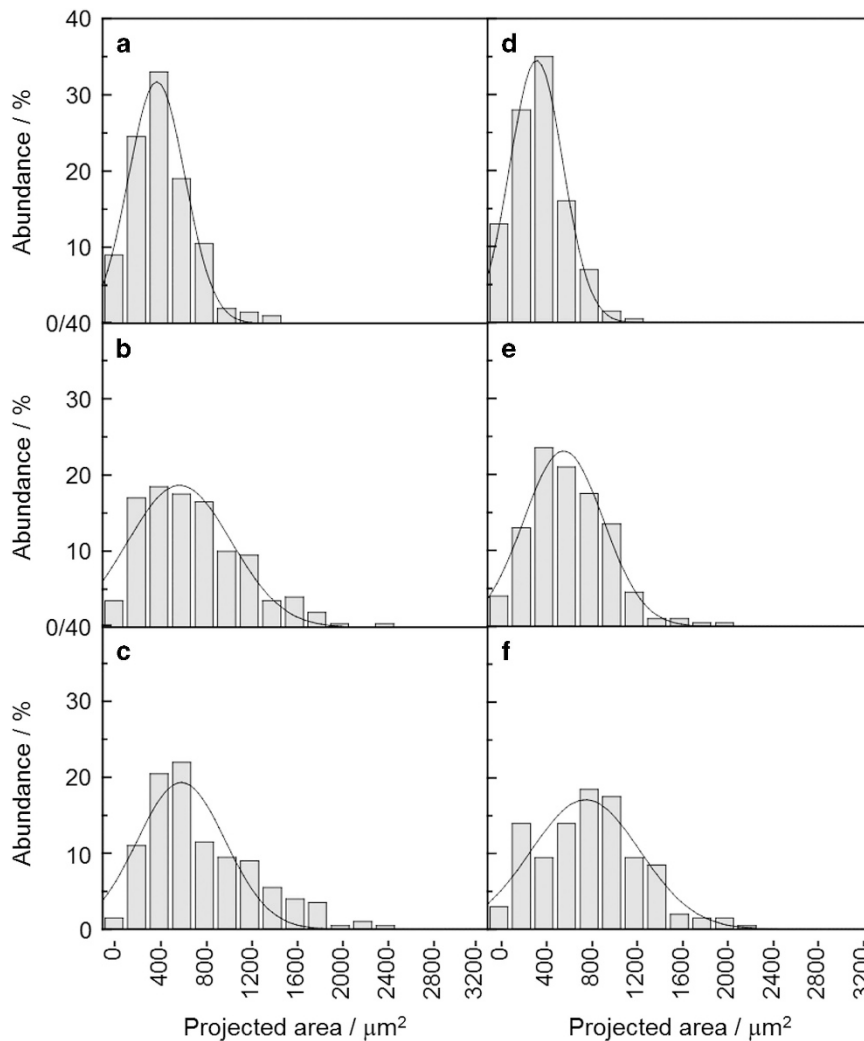


Figure 5 The abundance of the projected area of the L929 fibroblasts adhered on (a, d) a homogeneous PLA film, PLA-TiO_x composite films with a loading weight ratio of (b, e) 97/3 (wt/wt) and (c, f) 95/5 (wt/wt) under serum-free conditions. Culturing times were (a-c) 4 and (d-f) 24 h. The normal distribution curves are superimposed. The number of cells used for calculation of the abundance was 300 for each sample. PLA, poly(lactic acid); TiO_x, titanium oxide.

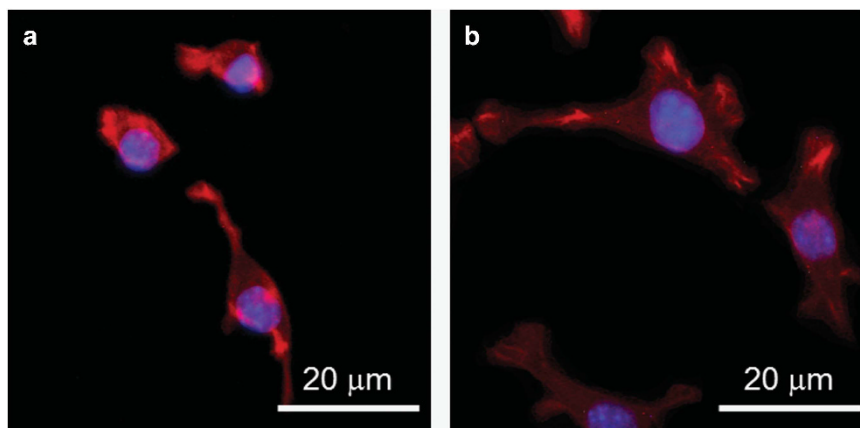


Figure 6 Fluorescent images of F-actin filaments (red) and nuclei (blue) of L929 fibroblasts cultured on a (a) homogeneous PLA film and (b) 95/5 (wt/wt) PLA-TiO_x composite film. PLA, poly(lactic acid); TiO_x, titanium oxide.

Table 1. This result implies that the chain dynamics within the surface region are restricted after the incorporation of TiO_x.

Cell behavior on the PLA-TiO_x composite films

Now, we will explore the effect of TiO_x on the cell behavior. Before the measurement, the stability of the scaffold films in a culture medium was examined on the basis of topographic observation using digital microscopy, as shown in Supplementary Figure S1 of the Supplementary Information (SI). To determine the toxicity of the hydrolyzed TEOT, cell adhesion and proliferation on the homogeneous TEOT_{hyd} film were examined. The cells behaved favorably on such surfaces, as shown in Supplementary Figures S2a and b in the SI. In addition, mortal cells were rarely observed on the homogeneous TEOT_{hyd} film according to the live/dead assay, as shown in Supplementary Figures S2c and d. This indicates that the hydrolyzed TEOT surface was not cytotoxic.

Figure 3 shows the phase contrast images of L929 fibroblasts on the homogenous PLA film in comparison with the 97/3 and 95/5 PLA-TiO_x composite films after culturing under serum-free conditions for 4 and 24 h. After culturing for 4 h, the number of cells adhered to the surface was greater for the PLA-TiO_x composite films than for the homogenous PLA film, as shown in Figures 3a–c. In addition, although the shape of the majority of the cells on the pristine film remained spherical, cells on the composite films spread out noticeably. These results clearly show that the fibroblasts were affected by the presence of TiO_x at the film surface during the initial adhesion process. Furthermore, this trend became more striking after culturing for 24 h, as shown in Figures 3d–f. This was particularly evident in the 95/5 composite film, where the observed spreading affected a more significant quantity of cells. Furthermore, mortal cells were rarely observed on all scaffold films examined, as shown in Supplementary Figure S3. The following two figures present a quantitative discussion of the above results based on the cell images in Figure 3.

Figure 4 shows the number of L929 cells attached to the various film surfaces. As shown in Figure 4a, the number of cells on the composite films initially was twice that on the PLA film. In addition, the difference in the number of cells between the composite and pristine films became larger after culturing for 24 h, as shown in Figure 4b. Therefore, it can be concluded that the TiO_x component at the surface enhanced the cell proliferation and the initial adhesion.

Generally, when fibroblast cells such as mouse L929 are cultured under appropriate conditions, they attach to a surface and then spread

out. The cell spreading on the PLA and PLA-TiO_x composite films was also evaluated based on the cell images. Figure 5 shows the abundance of the projected area of the L929 cells adhered on the films after culturing for 4 and 24 h. All histograms were fitted using simple Gaussian distributions. The peak position and full width at half maximum for each distribution in Figure 5 are summarized in Table 2. Both the peak position and full width at half maximum are larger for the composite films than for the PLA film. This indicates that the cells attached to the PLA composite films more strongly than to the homogenous PLA film, resulting in the cell spreading observed. These results indicate that the fibroblast cells recognize the TiO_x component introduced within the surface region of the PLA scaffold film, where the chain dynamics may be restricted due to the presence of the Ti-O-Ti network.

Cytoskeleton formation of cells adhered on the scaffolds

The tension induced by the formation of actin stress fibers inside the cells is one of the forces affecting the cell morphology and proliferation. To gain a better understanding of the structural response of cells attached to the scaffolds with a TiO_x component, the formation of F-actin filaments was observed by fluorescence microscopy using cells stained with fluorescent dyes after glutaraldehyde fixation. As shown in Figure 6, F-actin filaments clearly formed in the cells that adhered to the PLA-TiO_x composite surface. This was not the case for the homogenous PLA surface. Therefore, the introduction of TiO_x to the PLA film surface contributes to the formation of F-actin filaments, resulting in enhanced cell adhesion to the polymer scaffold surface.

To study the impact of serum proteins such as fibronectin and collagen on cell adhesion and proliferation, L929 fibroblast cells were cultured under serum conditions. Figures 7a–f show the phase contrast images of L929 fibroblasts on the homogenous PLA film in comparison with the 97/3 and 95/5 PLA-TiO_x composite films after culturing for 4 h and 3 days. The shape of the majority of the cells remained spherical regardless of the amount of TiO_x. However, the number of cells adhered to the surface was greater for the PLA-TiO_x composite films than for the PLA film regardless of culturing time. Figure 7g shows the culturing time dependence of the number of cells attached to the scaffold films, the so-called growth curves. The cell behavior was strongly dependent on the existence of TiO_x from around the middle to the end of the time period being examined. The number of cells on the 95/5 PLA-TiO_x composite film was larger than

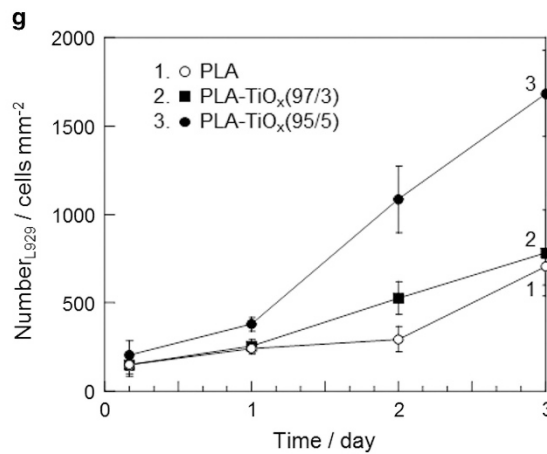
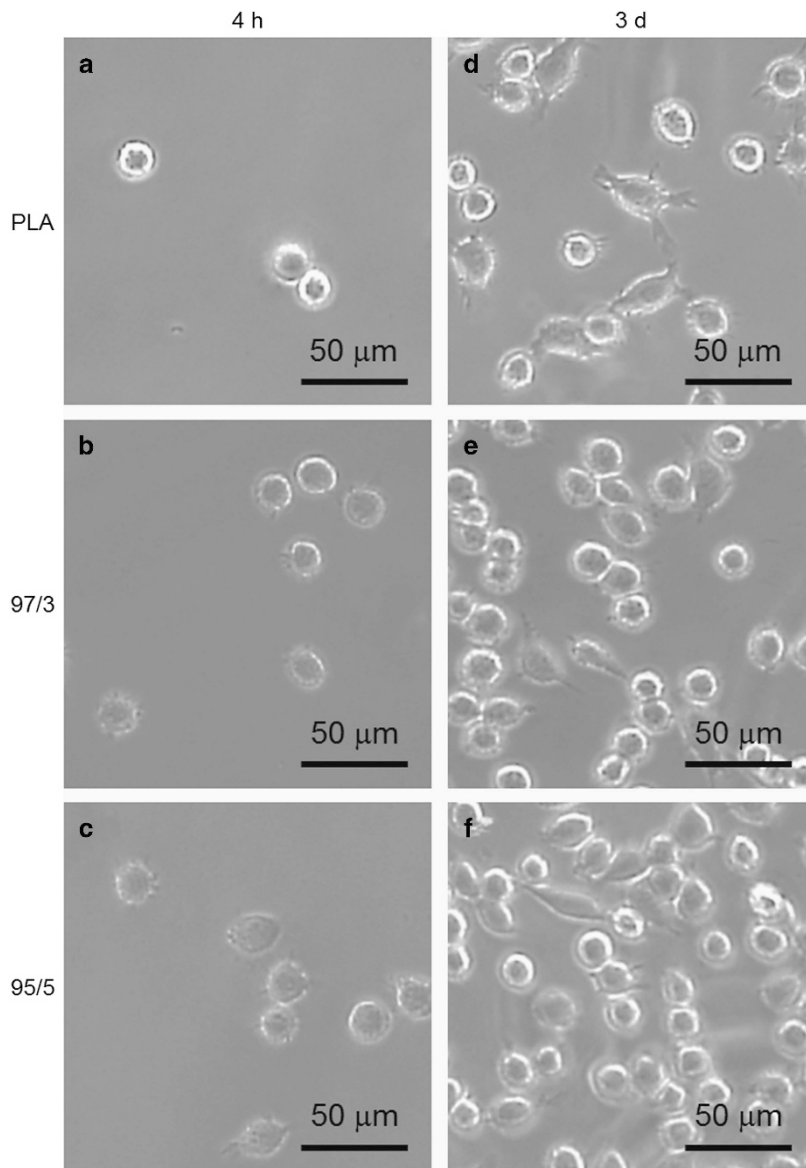


Figure 7 Phase contrast images of L929 fibroblasts adhered on (a, d) a homogenous PLA film, (b, e) 97/3 (wt/wt) PLA-TiO_x composite film and (c, f) 95/5 (wt/wt) PLA-TiO_x composite film after culturing for (a–c) 4 h and (d–f) 3 days under serum conditions. Scale bars correspond to 50 μm. (g) Growth curves of L929 fibroblasts on the homogeneous PLA film and PLA-TiO_x composite films. The data represent the mean ± s.d. PLA, poly(lactic acid); TiO_x, titanium oxide.

that on the other scaffold films. Hence, it is plausible that the TiO_x component that suppresses the surface mobility of the PLA also enhances the cell proliferation and the initial adhesion via the layer of serum proteins on the scaffold.

CONCLUSIONS

PLA-TiO_x composite films were successfully prepared using a simple sol-gel method with TEOT as a source of TiO_x. When the loading of TEOT in the film was <5% by weight, the surface was smooth on the nanometer scale. XPS analysis revealed that TiO_x was slightly segregated at the composite surface. Once TiO_x was incorporated into the PLA surface, initial cell adhesion and proliferation on the scaffolds were enhanced, accompanied by the formation of F-actin filaments due to the high biocompatibility of TiO_x derivatives, which led to the suppression of the chain surface mobility. In addition, the enhanced cell behavior was further observed under serum-present conditions. We believe that this facile surface modification method could be applied to many polymeric scaffolds and contribute to the development of highly functional novel scaffolds for cell culture.

ACKNOWLEDGEMENTS

This research was partly supported by JST SENTANKEISOKU (13A0004) and JSPS KAKENHI Scientific Research on Innovative Area 'New Polymeric Materials Based on Element-Blocks' (No. 25102535), 'Molecular Soft-Interface Science' (No. 23106716) programs, and a Grant-in-Aid for Scientific Research (B) (No. 24350061).

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