ORIGINAL ARTICLE

Enhanced proliferation of HeLa cells on PLLA-PCL and PLGA-PCL multiblock copolymers

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Bioabsorbable elastic materials are promising substrates for tissue engineering. A drawback of synthetic polymers as cell culture substrates is their low surface adhesion due to the lack of extracellular matrix. In this study, we found that the proliferation of HeLa cells was enhanced on films prepared from poly(L-lactide)-poly(ε-caprolactone) (PLLA-PCL) and poly(L-lactide-co-glycolide)-poly(ε-caprolactone) (PLGA-PCL) multiblock copolymers (MBCs) without any coatings or treatments. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays quantitatively showed that the proliferation of HeLa cells on the MBC films was enhanced compared with a film prepared from a PLLA-PCL random copolymer. Experiments using an anti-fibronectin antibody suggested that the adsorption of fibronectin on MBC films was essential for the attachment and proliferation of HeLa cells. The adsorption behavior of albumin was also different for the MBC and the random copolymer films. We previously reported that PLLA-PCL and PLGA-PCL MBCs synthesized from the corresponding diblock copolymer showed microphase separation. The microphase-separated morphology plays an important role in protein adhesion behavior and subsequent cell proliferation.
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INTRODUCTION

Poly(L-lactide) (PLLA) and its copolymers are the most intensively investigated biodegradable polymers.[1–7] The introduction of glycolide segments to form poly(L-lactide-co-glycolide) copolymer (PLGA) is known to improve hydrolysis rates.[8,9] Therefore, PLGA is used as a bioabsorbable surgical suture. Moreover, the introduction of low glass transition temperature (T_g) components such as caprolactone segments makes the resulting copolymer soft and elastic.[10–13] The synthesis and properties of PLLA-poly(ε-caprolactone) (PCL) random copolymers have been reported in the literature,[13,14] and PLLA-PCL random copolymers are now commercially available. We previously reported the synthesis and properties of PLLA-PCL and PLGA-PCL multiblock copolymers (MBCs) produced via self-polycondensation of the corresponding diblock copolymers.[15,16] The presence of each segment induced a microphase-separated morphology, which resulted in higher tensile moduli of these MBCs compared with that of a PLLA-PCL random copolymer.

One of the promising fields for the application of biodegradable polymers is tissue engineering.[4,17–22] Collagen, an extracellular matrix protein, is commonly used as a substrate for cell culture. As collagen is collected from animals or humans, the use of biodegradable synthetic polymers is potentially advantageous from the viewpoints of safety, cost and stable production. PLLA and its copolymers are good candidates as substrate materials because the degradation products

are safe and metabolizable. One drawback of synthetic polymers as cell culture substrates is the low adhesion of cells on their surface because of the lack of extracellular matrix proteins. Collagen coatings help improve the adhesion of cells on synthetic polymer films.[23] The introduction of polar functional groups, such as peptides derived from collagen, is also effective for cell adhesion.[24]

Several studies have discussed the effects of microphase-separated morphology on the interaction between cells or proteins and substrates. Segmented polyurethanes that form a microphase-separated surface have been used as blood compatible materials because of their good mechanical and moderately good antithrombotic properties.[25] Okano et al.[26,27] reported that hydrophilic–hydrophobic microdomain surfaces containing poly(2-hydroxyethyl methacrylate) suppressed platelet adhesion and activation. These examples show that microphase-separated surfaces are effective in reducing platelet adhesion, which is required for developing blood compatible devices and implant materials. It is noted that the adsorption of protein occurs prior to the cell proliferation on substrates.[28] Adsorption behaviors on microphase-separated surfaces have also been reported in the literature.[29–33] In the case of segmented polyurethanes, plasma proteins preferentially adsorbed onto apolar polybutadiene soft segment microdomains.[29]

In this study, we report the growth of HeLa cells on films prepared from PLLA-PCL and PLGA-PCL MBCs and a PLLA-PCL random

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copolymer. HeLa cells were cultured on PLLA-PCL and PLGA-PCL MBC films without any treatment, such as collagen coating. Cell proliferation was clearly enhanced on the microphase-separated MBC films compared with that on the PLLA-PCL random copolymer film. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to quantify differences in proliferation and cell viability. The effect of fibronectin on cell adsorption to the films was examined through experiments using anti-fibronectin antibody.

EXPERIMENTAL PROCEDURE
Materials
PLLA-PCL MBC and PLGA-PCL MBC were synthesized according to a previously described procedure using organic catalysts.[15,16] The molecular weight of the PLGA-PCL MBC was controlled by the addition of benzyl-protected PLGA-PCL diblock copolymer during the self-polycondensation of PLGA-PCL diblock copolymer. PLLA-PCL random copolymer was purchased from BMG Inc. (Kyoto, Japan). Table 1 presents the list of the characterization data for the copolymers used in this study. The MBC and random copolymer films were prepared on a glass plate by casting a chloroform solution of the copolymer. The solution of the copolymer (PLLA-PCL MBC: 0.15 g, PLGA-PCL MBC: 0.3 g, PLLA-PCL random copolymer: 0.4 g) dissolved in chloroform (4 ml) was cast on a glass plate. After drying at room temperature at atmospheric pressure, the film on the glass plate was dried under vacuum at room temperature for 12 h. The film was removed from the glass plate by immersing the plate into water. The removed film was wiped and dried under vacuum at 40 °C for 12 h. The thicknesses of the MBC and random copolymer films were in the range of 20–30 μm and 50–60 μm, respectively. Atelocollagen membrane was purchased from Koken Co., Ltd. (Tokyo, Japan). All other materials were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan) and were used as received. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Tissue culture of HeLa cells was performed according to a previously described method but with a little modification.[34] HeLa cells in a 3.5-cm-diameter dish were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Tokyo, Japan) containing 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin and streptomycin (PS, Nacalai Tesque, Kyoto, Japan) at 37 °C under 5% CO_2 with humidity. The MTT reduction assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA), and the assay was performed per the manufacturer's protocol.

Cell culture experiments
A copolymer film with a size of 2.0×2.0 cm^2 was sterilized with 70% ethanol and washed with phosphate-buffered saline (PBS, pH = 7.4) and DMEM containing 10% FBS and 1% PS. The film was placed on a 3.5-cm dish and immobilized using sterilized glass plates. The medium (DMEM containing FBS and PS, 1.5 ml) was added to the dish, and 150 μl of the cells suspended in the medium (3000 cell/μl) were spread onto the film. After incubation for 48 h, the film was taken out from the dish and washed gently with water. The film surface was observed using an optical microscope.

MTT assay
Film with a size of 0.8×0.8 cm^2 was sterilized with 70% ethanol and washed with PBS and DMEM containing 10% FBS and 1% PS. The film was placed in a 96-well plate and fixed with a sterilized wire. Approximately 1500 HeLa cells in DMEM containing FBS and PS (99 μl) were added to the 96-well plate, and the mixture was incubated at 37 °C for 24, 48 and 72 h in an atmosphere of 5% CO_2–95% air. After a certain period of incubation, 10 μl of the MTT reagent was added to each well and allowed to stand for 3.5 h at 37 °C. Then, the medium was removed, and 100 μl of crystal dissolving solution (isopropanol containing HCl) was added and the plate was shaken for 30 min at 22 °C to dissolve the formed formazan. The polymer films and the wire were removed from the 96-well plate, and the solution was transferred to a test tube and centrifuged for 1 min at 20 °C (5000 r.p.m.) to remove the precipitate. The supernatant (70 μl) was transferred to a 96-well plate, and the absorbance at 570 nm was measured. A control experiment without HeLa cells was also conducted to estimate the background of the absorbance at 570 nm.
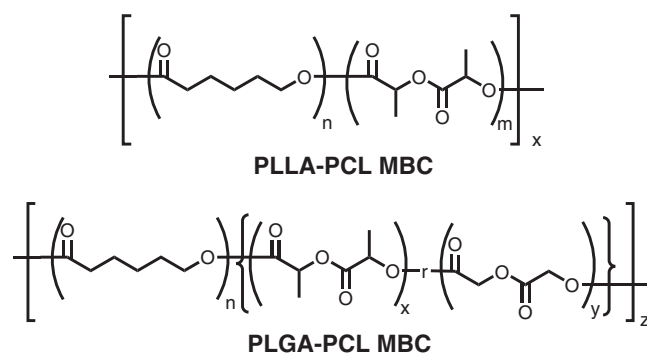
Fibronectin adhesion test
Film with a size of 1.5×1.5 cm^2 was immersed in 1.5 ml of DMEM containing 10% FBS at 37 °C. After 24 h, the medium was removed, and the film was washed with 1 ml of an immunostaining (IS) buffer (PBS solution containing 5% glycerol and 10% BSA). The film was immersed in 1.0 ml of IS buffer for 5 min at room temperature. After the IS buffer was removed, 40 μl of primary antibody (mouse monoclonal anti-fibronectin (clone FN12-8), Takara, Japan) diluted 100-fold (final concentration 4 μg ml^{-1}) with the IS buffer was placed onto the film and allowed to stand at room temperature. After 20 min, the film was washed with the IS buffer, and 40 μl of secondary antibody (goat anti-mouse IgG conjugated with Alexa Flour 488, Molecular Probes) diluted 400-fold (final concentration 5 μg ml^{-1}) with the IS buffer was placed onto the film in a dark place. The excess secondary antibody was removed, and the film was washed with 1 ml of the IS buffer. Images of the film surface were taken using a confocal microscope.

Cell culture inhibition experiments with anti-fibronectin antibody
Film with a size of 1.5×1.5 cm^2 was immersed in 1.5 ml of DMEM containing 10% FBS and 1% PS at 37 °C. After 24 h, the medium was removed, and the film was washed with 1 ml of PBS. The film was immersed in 1.0 ml of PBS containing 2% BSA for 5 min at room temperature. After the PBS was removed, 40 μl of mouse anti-fibronectin antibody diluted 100-fold with the IS buffer was placed onto the film. After 20 min, the anti-fibronectin antibody in the medium was removed, and the film was washed with 1 ml of the IS buffer. The film was placed on a polystyrene dish, and HeLa cells (270 000 cells) in serum-free DMEM (1.5 ml) were added to the dish. After incubation at 37 °C for 24 h, the medium was removed, and the film was washed with PBS and observed using an optical microscope.

Albumin adhesion test
BSA was dissolved in PBS at a concentration of 0.5 mg ml^{-1}. The copolymer-coated glass plate was immersed in the BSA solution at 37 °C for 2 h. After

Table 1 Characterization data for the copolymers as a substrate for HeLa cell culture

Copolymer	DP_{CL}[a]	DP_{LA}[a]	DP_{GA}[a]	M_w[b] ($\times 10^4$ g mol^{-1})	M_w/M_n[b]
PLLA-PCL MBC	27	25	—	57.0	2.3
PLGA-PCL MBC	25	30	3	12.3	2.6
PLLA-PCL random	—[c]	—[c]	—	40.0	1.8

Abbreviations: PLGA-PCL MBC, poly(L-lactide-co-glycolide)-poly(ε-caprolactone multiblock copolymers; PLLA-PCL MBC, poly(L-lactide)-poly(ε-caprolactone multiblock copolymers.
[a]Determined by ^{1}H NMR measurements in CDCl$_3$.
[b]Determined by GPC measurements on the basis of polystyrene standards.
[c]The molar ratio (lactide:caprolactone) of PLLA-PCL random copolymer is 53:47.

PLLA-PCL MBC

PLGA-PCL MBC

Figure 1 Structure of poly(L-lactide)-poly(ε-caprolactone) (PLLA-PCL) and poly (L-lactide-co-glycolide)-poly(ε-caprolactone) (PLGA-PCL) multiblock copolymers.

being gently washed with water, the surface of the copolymer film was examined using AFM measurements in water.

Measurements

^{1}H and ^{13}C NMR spectra were recorded using a JEOL JNM-ECX 500 NMR spectrometer (Tokyo, Japan). Gel permeation chromatography (GPC) measurements (Shodex KF802.5 and KF806M columns, Tokyo, Japan) were conducted using chloroform as an eluent, and the average molecular weights were calculated on the basis of polystyrene standards. Tapping mode atomic force microscopy (AFM) measurements were performed using a NanoNavi S-image SPM system (Hitachi High-Tech Sci. Co., Tokyo, Japan) using a cantilever with a force constant of $\sim 15\,N\,m^{-1}$. Optical microscopy measurements were performed using a VK-X200/210SP optical laser microscope (Keyence, Tokyo, Japan). Confocal microscopy measurements were performed using a Zeiss LSM780 confocal microscope (Carl Zeiss, Inc., Germany).

RESULTS AND DISCUSSION

PLLA-PCL and PLGA-PCL MBCs were synthesized according to a method previously reported in the literature.[15,16] Figure 1 shows the structure of the MBCs examined in this study. The characterization data for all the copolymers are summarized in Table 1. The composition ratio of the hard (PLLA or PLGA) and soft (PCL) components of all the copolymers is ~ 50:50. It has been reported that PLLA-PCL MBCs and PLGA-PCL MBCs exhibit a microphase separation in contrast to PLLA-PCL random copolymers.[15,16] Figure 2 shows AFM images of the cast films of the copolymers. Microphase-separated surfaces composed of soft (PCL) and hard domains (PLLA or PLGA segments) were observed for the PLLA-PCL MBC and PLGA-PCL MBC films. The bright and dark areas represent the soft (PCL) and hard (PLLA or PLGA) domains, respectively. In addition to the round-shaped domains, dark fibrous domains were observed on the PLLA-PCL MBC film. This unique morphology is caused by the crystallization of PLLA segments.[15] The PLLA-PCL random copolymer did not show phase-separated domains, implying that the PLLA and PCL segments in the random copolymer are miscible with each other. As all films were prepared by solution casting, the surface of the films was flat and smooth. The average values of surface roughness (Ra) of the films shown in Figure 2a–c were 2.4, 2.3 and 1.8 nm, respectively. To evaluate more macroscopic roughness, the film surface was observed using an optical microscope, as shown in Figure 3. The surface of the random copolymer film was slightly rougher than that of the PLLA-PCL MBC film. The Ra of

the films in 100 μm square areas was in the range of 0.08–0.20 μm (PLLA-PCL MBC) and 0.15–0.40 μm (random copolymer). Because the difference in roughness was small, we assume that the effect of this roughness on cell culture in this study is negligible.

Owing to the lack of cell-adhesive proteins, artificial materials generally show poor cell-adhesive properties. Collagen coating is often required to culture cells on artificial materials. In this study, HeLa cells, the most widely used human cell line, were cultured on PLLA-PCL copolymer films without a collagen coating, as shown in Figure 4. After 48 h, good cell proliferation was observed when cells were cultured on collagen, PLLA-PCL and PLGA-PCL MBC films (Figure 4a–c). The number of cells on the collagen film is clearly higher than those on the multiblock copolymers. As cell colonies were observed on the PLLA-PCL MBC film, accurate counting of the number of cells was difficult. The reason for the colony formation is not clear, and we assume that the crystalline PLLA domain may affect the formation of these colonies. In contrast to the MBC films, the number of HeLa cells cultured on the PLLA-PCL random copolymer film was clearly lower (Figure 4d). The poor cell adhesion tendency on the PLLA-PCL random copolymer is consistent with results reported in the literature.[23] The difference in the proliferation indicates that the microphase-separated surface of MBC films exhibits beneficial properties for HeLa cell adhesion without collagen coating. Although the molecular weight of PLGA-PCL MBC shown in Figure 4c is lower than that of other polymers, similar proliferation behavior was observed in the HeLa cell culture on the high-molecular-weight PLGA-PCL MBC film (Supplementary Figure S1). An MTT assay was performed to quantitatively evaluate HeLa cell proliferation. The absorbance at 570 nm was monitored to evaluate the amount of formazan formed by the reduction of MTT reagent by mitochondrial oxidoreductase. Figure 5 shows the results of the MTT assays of HeLa cells cultured on various films. On PLLA-PCL and PLGA-PCL MBC films, the absorbance at 570 nm increased with time, which implies that the number of HeLa cells increased with increasing incubation time. It should be noted that the cell proliferation on MBC films seems similar to that on a collagen film in these MTT experiments. This similarity is caused by the small number of applied HeLa cells on the films in the MTT experiments. On the PLLA-PCL random copolymer film, the absorbance at 570 nm did not increase during the examined period and the difference in the absorbance was noticeable after 72 h of culturing. Therefore, we concluded that there
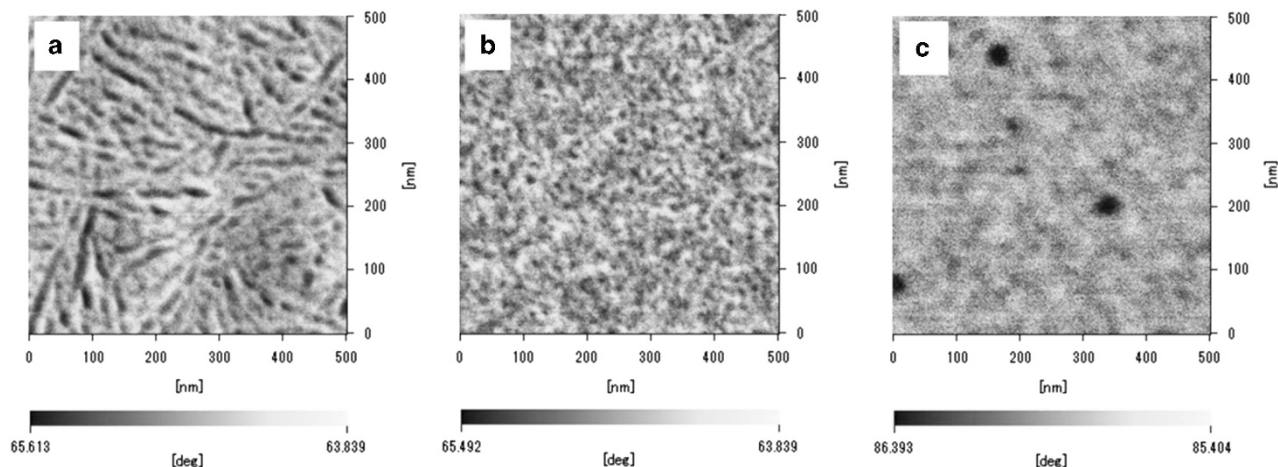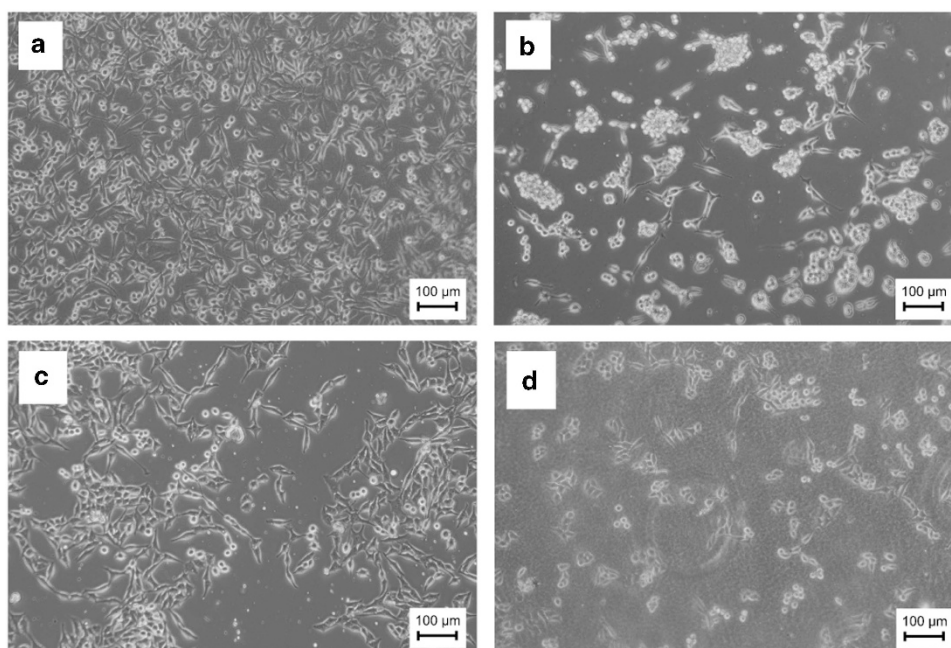

Figure 2 AFM phase images of PLLA-PCL MBC (**a**), PLGA-PCL MBC (**b**), and PLLA-PCL random copolymer (**c**) films. A full colour version of this figure is available at the *Polymer Journal* journal online.
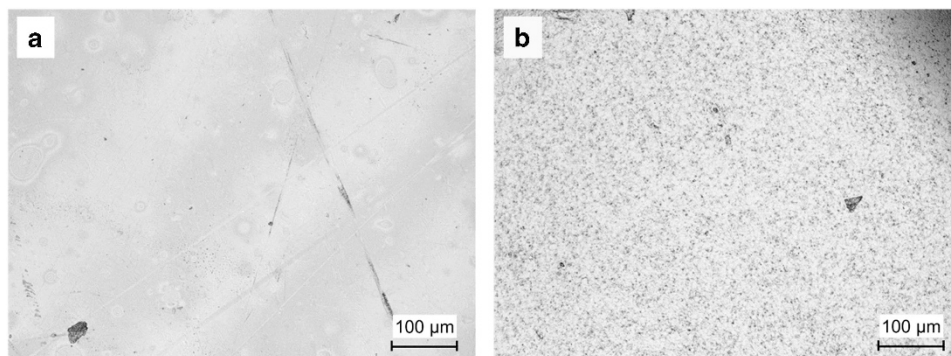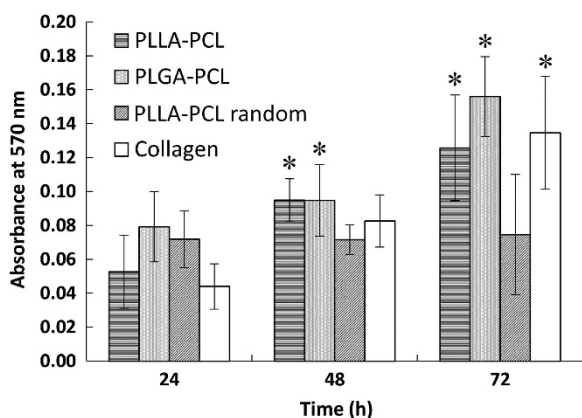
**Figure 3** Optical microscopy images of PLLA-PCL MBC (**a**) and PLLA-PCL random copolymer (**b**) films.

Figure 4 HeLa cells cultured on copolymer films after incubation for 48 h. Collagen (**a**), PLLA-PCL MBC (**b**), PLGA-PCL MBC (**c**) and PLLA-PCL random copolymer (**d**). A full colour version of this figure is available at the *Polymer Journal* journal online.

Figure 5 MTT assays to determine HeLa cell proliferation when cultured on the copolymer films ($n=10$). *Significant difference ($P<0.05$) when compared with the PLLA-PCL random copolymer.

is a significant difference in the HeLa cell culture between the MBCs and the random copolymer films. Cell adhesion proteins (for example, fibronectin) are known to first adhere onto the surface of substrates before cell proliferation.[28] The microphase-separated surface of MBC films affects the adsorption of cell adhesion proteins, such as fibronectin, which resulted in the difference in cell culturing behavior between multiblock and random copolymer films.

Fibronectin is a cell-adhesive protein that plays an important role in initial cell attachment to substrates. The adsorption behavior of fibronectin on the films examined in this study was monitored using an anti-fibronectin antibody. After incubation in a medium containing FBS and fibronectin at 37 °C for 24 h, an anti-fibronectin antibody was spread onto the film to induce an antigen–antibody interaction. Then, secondary antibody marked with a fluorescent probe was added to monitor the adsorption of fibronectin on the film. As shown in Figure 6, the collagen film showed strong fluorescence, which implies strong adsorption of fibronectin because collagen binds fibronectin to form the extracellular matrix.[35] Many fluorescent spots were observed
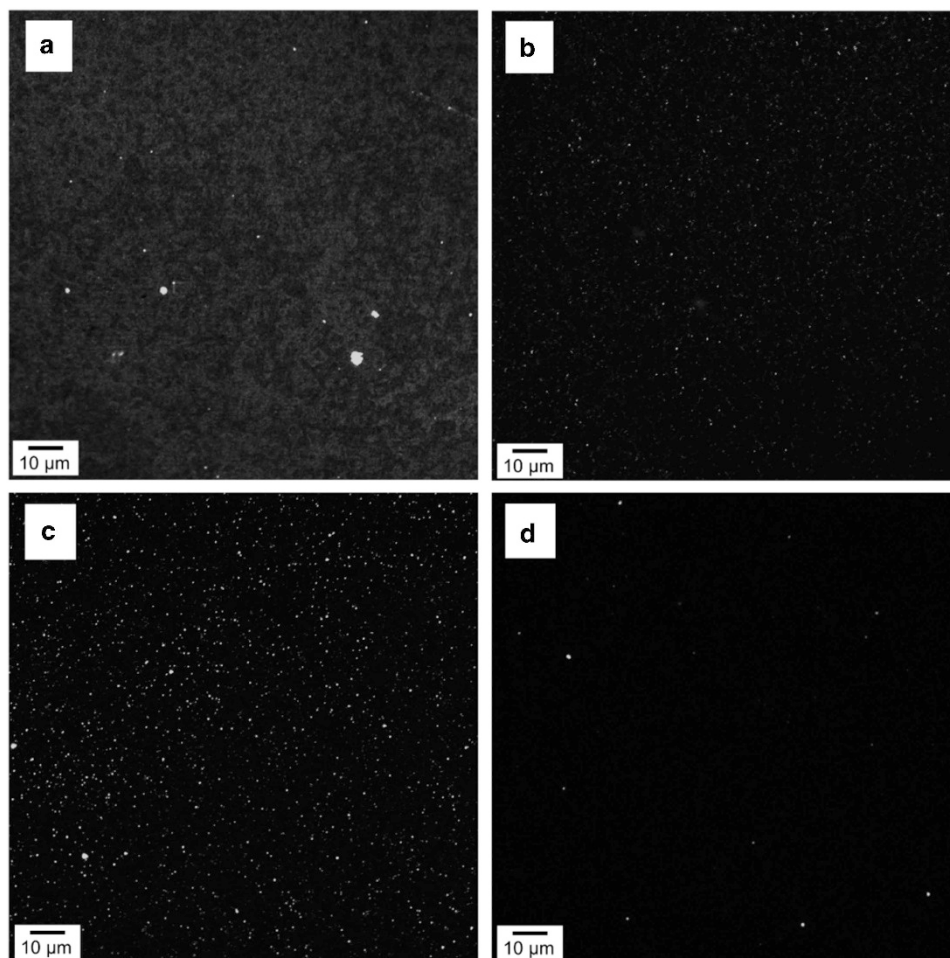
Figure 6 Adsorption of fibronectin on collagen (**a**), PLLA-PCL MBC (**b**), PLGA-PCL MBC (**c**), and PLLA-PCL multiblock copolymer (**d**) films. A full colour version of this figure is available at the *Polymer Journal* journal online.

on the surface of the two MBC films. By contrast, the number of fluorescent spots was significantly lower on the surface of the PLLA-PCL random copolymer. Because the composition ratio of PLLA and PCL in the multiblock and random copolymers is approximately the same, the difference in the adsorption of fibronectin is caused by the difference in surface morphology; the microphase-separated surface is preferable for the adsorption of fibronectin. The difference in the adsorption behavior of fibronectin on the films influences the initial attachment of the cells on the films. The cell attachment on the MBC films resulted in enhanced cell proliferation, as evidenced by the MTT assay.

To investigate the influence of fibronectin adsorbed on a film surface on cell culture, cell culture inhibition experiments using anti-fibronectin antibody were performed. Because the anti-fibronectin antibody used in this study recognizes the cell adhesive region of fibronectin, masking fibronectin in the medium with this antibody results in decreased cell binding. In the first step, the PLGA-PCL MBC film was immersed in a medium containing serum (FBS, 10%) and PS (1%) to adsorb fibronectin. The adsorbed fibronectin on the film was blocked by anti-fibronectin antibody in the second step. Finally, HeLa cells were cultured on the pretreated films in serum-free medium. Adsorbed cells on the film were observed using an optical microscope (Figure 7). The number of cells on the film treated with the antibody was found to be drastically decreased (Figure 7b) compared with those

on the untreated film (Figure 7a). This result suggests that blocking the adsorbed fibronectin caused cell adhesion inhibition. This result also implies that fibronectin adsorbed on the microphase-separated surface of PLGA-PCL MBC is essential for cell culture.

Because the concentration of albumin in serum is significantly higher than that of fibronectin in serum, albumin should be adsorbed first on the surface of the copolymer films. The adsorption of albumin on the films was observed using AFM measurements in water (Figure 8). Many albumin grains were observed on the PLGA-PCL MBC film. The surface of the PLLA-PCL random copolymer film was relatively smoother, and the number of grains was significantly lower than that observed on the PLGA-PCL MBC film. It is clear that the microphase-separated morphology of the PLGA-PCL MBC film affected the adsorption behavior of albumin. Adsorbed proteins have been reported to be competitively exchanged by a subsequently arriving protein, which is called the Vroman effect.[36] In this study, the adsorbed albumin was exchanged with fibronectin, which enabled cell attachment on the MBC films. We assume that the rough and granular surface of adsorbed albumin on the MBC film facilitated this competitive exchange. The reason for the unique adsorption behavior of albumin onto the PLGA-PCL MBC film was discussed on the basis of the following experiments. The albumin adhesion test at pH 4.7, which is the isoelectric point of albumin, was performed to examine the effect of the negative charge of albumin at pH 7.4. As shown in
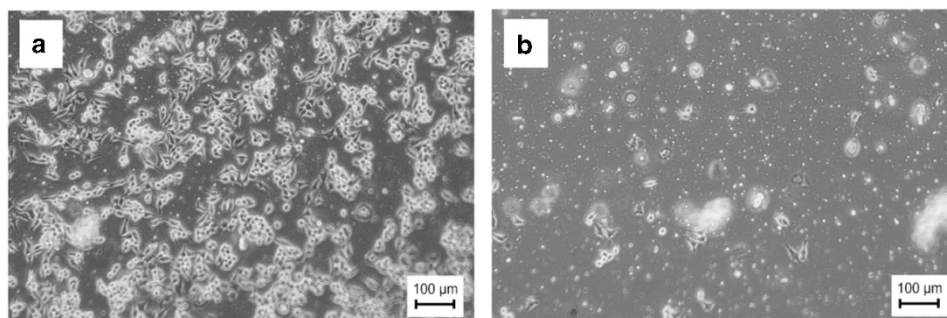
Figure 7 HeLa cells cultured on PLGA-PCL MBC films without anti-fibronectin antibody treatment (**a**) and with anti-fibronectin antibody treatment (**b**). A full colour version of this figure is available at the *Polymer Journal* journal online.
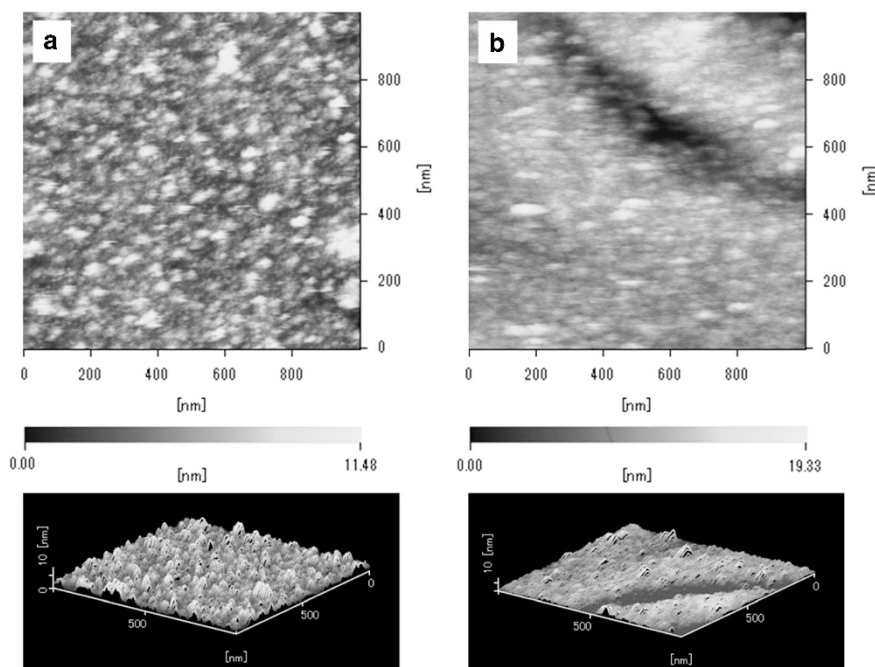
Figure 8 AFM images of albumin adsorbed on PLGA-PCL MBC film (**a**) and PLLA-PCL random copolymer film (**b**). A full colour version of this figure is available at the *Polymer Journal* journal online.

Supplementary Figure S2, the AFM image of the albumin-adsorbed film at pH 4.7 was very similar to that at pH 7.4. Therefore, the effect of the negative charge of albumin on the adsorption onto PLGA-PCL MBC film was negligible. Contact angles of PLGA and PCL homopolymers were evaluated as 76 and 79 degrees, respectively. It is difficult to attribute the preference of the albumin adsorption to the hydrophobicity due to the small difference in these contact angles. Moreover, the grain size observed in Figure 8a is ∼3 times larger than the size of the domain observed in Figure 2b. Currently, we cannot conclude the reason for the unique adsorption behavior of albumin onto PLGA-PCL MBC film. Additional experiments exploring the initial adsorption should provide information about the relationship between albumin adsorption and the microphase-separated domains.

CONCLUSION

HeLa cell adhesion and proliferation were enhanced on the PLLA-PCL and PLGA-PCL MBC films compared with that on the PLLA-PCL random copolymer film. MTT assays enabled quantitative estimation

of the enhanced proliferation. According to the experiments conducted using an anti-fibronectin antibody, the adsorption of fibronectin on the MBC films played a key role in the attachment and proliferation of HeLa cells. Adsorption of albumin, which occurred first on the film during the cell culture, was monitored via AFM measurements in water. A unique granularity, which facilitated the competitive exchange between albumin and fibronectin, was observed on the PLGA-PCL MBC film.

As described in the introduction, microphase-separated surfaces composed of hydrophilic and hydrophobic segments suppressed platelet adhesion and activation.[26,27] In this study, all components were hydrophobic and the hard (PLLA or PLGA) and soft (PCL) segments induced microphase separation. The microphase-separated morphology played a key role in cell adhesion and proliferation. We have preliminarily examined the culture of fibroblast cells on the MBC films. A similar cell proliferation was observed without any coatings or treatments. As elastic bioabsorbable materials can be implanted directly with cultured cells, PLLA-PCL and PLGA-PCL MBCs are potentially promising bioabsorbable substrate candidates for tissue engineering.

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