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Time-dependent effects of pre-aging polymer films in cell culture medium on cell adhesion and spreading

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Abstract We have tested the hypothesis that cell adhesion and spreading on polymer films are influenced by the amount of time that the polymer films are pre-aged in cell culture medium. Cell adhesion and spreading were assessed after a 6-h culture on poly(D,L-lactic acid) (PDLLA) films that had been pre-aged in cell culture medium for 30 min, 1, 3 or 7 d. Cell adhesion and spread area were enhanced as the duration of pre-aging PDLLA films in cell culture medium was increased. Materials characterization showed that the hydrophobicity and surface morphology of the PDLLA films changed with increasing length of pre-aging time. These results suggest that cell adhesion and spreading are sensitive to the time-dependent changes in PDLLA hydrophobicity and surface morphology that occur during exposure of the polymer to cell medium for different lengths of time. These results demonstrate that cell response to a degradable, biomedical polymer can change as a function of the amount of time that the polymer is exposed to physiological medium.

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1 Introduction

When a biodegradable polymer, such as poly(D,L-lactic acid) (PDLLA), is placed in aqueous, physiological medium, many events occur that change the properties of the polymer. The polymer absorbs water-causing swelling [1, 2], the polymer begins to degrade [3–5] and proteins adsorb onto the polymer surface [6]. These changes affect surface properties of the polymer such as chemistry, modulus, hydrophilicity, roughness and morphology. In addition, these changes in the polymer properties are time-dependent and can become more severe as the length of time that the polymer is placed in a physiological environment is increased. For instance, both polymer swelling and polymer degradation will increase with time [1, 2]. Furthermore, the amount of protein adsorbed to a surface can change over time, the particular proteins that are adsorbed to a surface can change over time and the conformation of adsorbed proteins can change over time [3–8]. Since the physical properties of a polymer will change with time during incubation in physiological medium, it is plausible that cell behavior on a polymer surface will be influenced by the length of polymer exposure to physiological medium. Thus, we have tested the hypothesis that cell response to a polymeric material is affected by the amount of time that the polymer has been incubated in physiological medium.

The hypothesis was tested in vitro using polymer films and a cell line. Poly(D,L-lactic acid) (PDLLA) was chosen for the test polymer because it is biocompatible, degradable and commonly used in tissue engineering applications. An osteoblast cell line was used to test cell response because degradable polymers like PDLLA are commonly used for bone tissue engineering applications [9]. The MC3T3-E1 cell line was chosen because it is a well-characterized

murine pre-osteoblast cell line, which serves as an osteoblast model [9, 10]. Cell culture medium served as the physiological medium for incubating the PDLLA films. Finally, cell adhesion and morphology were the responses that were measured because these cell parameters are critically linked to cell function [11, 12]. Thus, PDLLA films were pre-aged in cell culture medium for various times between 30 min and 1 week. MC3T3-E1 osteoblasts were seeded on the pre-aged PDLLA films and adhesion and spreading were assessed after a 6 h culture time. The results showed that cell adhesion and spreading were influenced by the amount of time that the PDLLA had been pre-aged in cell culture medium.

2 Materials and methods

2.1 Preparation of polymer films

Thin films of PDLLA [poly(D,L-lactic acid); relative molecular mass = M.W. = 330,000–600,000 g/mol; Polysciences, Warrington, PA] were made using a home built flowcoater [13, 14]. PDLLA is a biodegradable, amorphous polymer with a glass transition temperature at 55–60 °C. For flowcoating, a 1% PDLLA solution (by mass, in chloroform) was injected into the space between an angled (4.5°) glass slide positioned 0.2 mm over a flat substrate (Low-e slide; Kevley Technologies, Chesterland, OH). A motorized stage moved the substrate beneath the slide such that the PDLLA solution was evenly spread across the substrate. The solvent rapidly evaporated from the spread PDLLA solution leaving behind a 25 mm × 25 mm thin film. The polymer films were annealed for 24 h at 120 °C under nitrogen (above glass transition temperature). This specific protocol for preparing PDLLA films was used because it yields films that do not delaminate when incubated in aqueous medium [15]. Thickness of annealed films was determined by scratching the films and measuring scratch depth by atomic force microscopy (162 nm; standard deviation = SD = 12 nm; $n = 3$).

2.2 Pre-aging polymer films for cell experiments

Forty-eight PDLLA films were prepared, sterilized in 70% ethanol (by mass) for 5 min, rinsed with serum-free medium (SFM) and placed in 150 cm^2 Petri dishes (3 slides per dish) with 60 mL of medium. Half of the films (24) were pre-aged in medium with serum (MWS) and half were pre-aged in SFM. SFM was Eagle's minimum essential medium (Cambrex Bio Science, Walkersville, MD) with 0.060 mg/mL kanamycin sulfate (Sigma, Inc., St. Louis, MO). MWS was SFM supplemented with 10% (by volume) fetal bovine serum (Gibco, Rockville, MD). Films were placed in a CO_2 incubator (5% volume fraction CO_2 and 100% relative humidity) and were pre-aged for 30 min, 1, 3 or 7 d. An identical pre-aging protocol was followed using 48 glass slide controls where half were pre-aged in MWS and half were pre-aged in SFM for the same incubation times. Glass was used as a control because it will not swell or degrade.

2.3 Cell culture

Established protocols for culture of MC3T3-E1 cells (Riken Cell Bank, Hirosaka, Japan) were followed [16]. Cells were cultured in tissue culture polystyrene flasks (75 cm^2 surface area) at 37 °C in a fully humidified atmosphere at 5% CO_2 (volume fraction) in alpha-modification of Eagle's minimum essential medium (α-MEM, Cambrex Bio Science, Walkersville, MD) supplemented with 10% volume fraction fetal bovine serum (Gibco, Rockville, MD) and 0.060 mg/mL kanamycin sulfate (Sigma, Inc., St. Louis, MO). Medium was changed twice weekly and cultures were passaged with 2.5 mg/mL trypsin (0.25% mass fraction) containing 1 mmol/L EDTA (Gibco, Rockville, MD) once per week. Cultures of 80% confluent MC3T3-E1 cells were used for all experiments.

2.4 Cell adhesion and cell spreading

For cell seeding, 750,000 MC3T3-E1 cells were added to each dish of pre-aged polymer or control glass specimens (5,000 cells/cm^2). For the cell adhesion and spreading studies, there were 16 treatments with 6 specimens per treatment yielding a total of 96 specimens {[2 medium types (MWS or SFM)] * [2 surface types (PDLLA or glass)] * [4 pre-aging times (30 min, 1, 3 or 7 d)] * [6 specimens per treatment] = 96}. Note that specimens were pre-aged sequentially such that all specimens were ready for cell seeding at the same time. For instance, the 7 d pre-aged samples were placed in medium 7 d before the cell seeding while the 30 min pre-aged samples were not placed in medium until 30 min before the cell seeding. After adding cells to the specimens in the dishes, the cells were gently mixed and the dishes were left undisturbed in the hood for 30 min to allow cell attachment [17]. The pH of the cell medium did not change significantly during this time. After 30 min, all dishes were moved to the CO_2 incubator for a 6-h incubation. Cells were incubated on substrates for 6 h in all cases. For specimens that were pre-aged in SFM, the 6 h cell adhesion experiment was performed in SFM. For the specimens that were pre-aged in

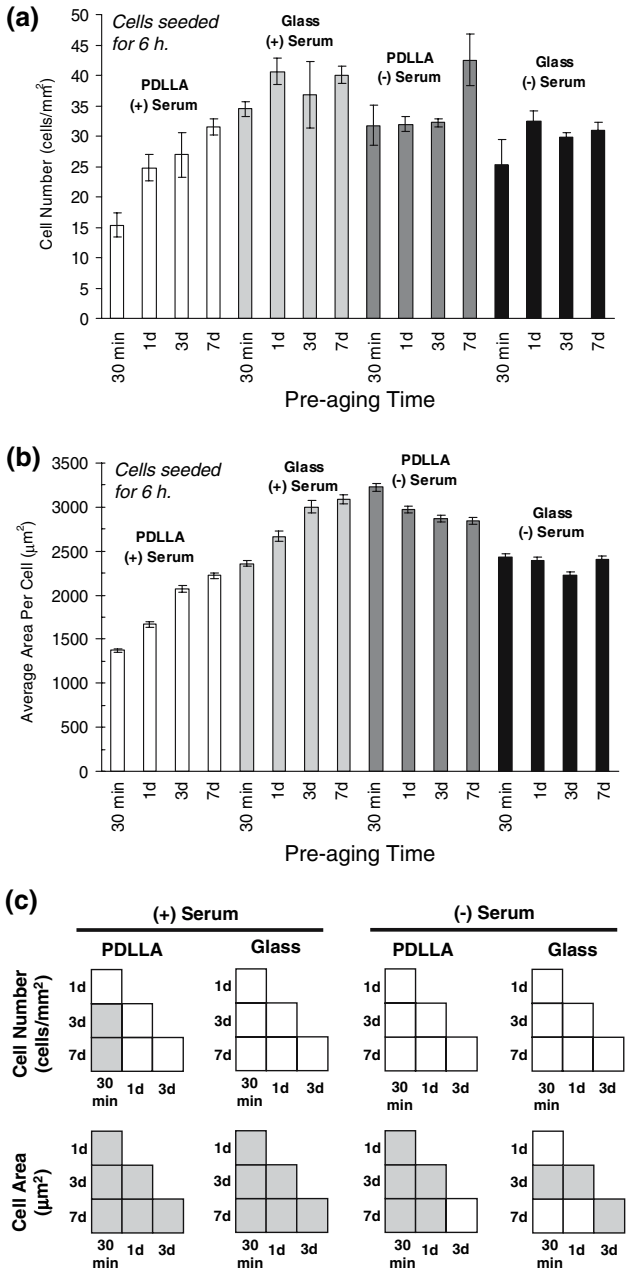

Fig. 1 (**a**) Cell adhesion on surfaces pre-aged in cell medium. Glass or PDLLA substrates were pre-aged in cell medium for times indicated on the bottom axis. White bars are PDLLA films pre-aged in MWS, light gray bars are glass slides pre-aged in MWS, dark gray bars are PDLLA films pre-aged in SFM and black bars are glass slides pre-aged in SFM. After pre-aging, cells were seeded on the substrates and incubated for 6 h in all cases. Cell culture was performed in the same medium (with or without serum) as was the pre-aging for each condition. Cell adhesion was assessed with automated fluorescence microscopy. Each bar is the average of data from 6 substrates ($n = 6$) and error bars are SD of the mean. (**b**) Cell spreading on surfaces pre-aged in cell medium. This experiment is similar to Panel (a) and the difference is that cell area was determined by automated fluorescence microscopy (instead of cell number). Cells were seeded for 6 h in all cases. Each bar is the mean cell area of more than 900 cells pooled from all 6 specimens for each treatment. Error bars represent the SD of the mean. (**c**) ANOVA with Tukey's test for multiple comparisons was used to analyze the cell adhesion and spreading data in Panels (a) and (b). Shaded boxes indicate significant differences ($p < 0.05$)

MWS, the cell adhesion experiment was performed in MWS.

Cells were seeded on substrates for 6 h in all cases, fixed for 5 min (0.5% mass fraction Triton X-100, 4% mass fraction paraformaldehyde, 5% mass fraction sucrose, 1 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$ in phosphate buffered saline, pH 7.4) and post-fixed for 20 min (same as fix but without Triton X-100). Fixed cells were fluorescently stained for 1 h with 6 µmol/L DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) and 2 µmol/L Texas Red C_2-maleimide (both from Molecular Probes, Eugene, OR) in phosphate buffered saline. DAPI stains cell nuclei blue and Texas Red C_2-maleimide stains cell membranes red [18]. Stained cells were mounted with a coverslip in Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA).

2.5 Automated fluorescence microscopy

Cell number and area for each specimen were determined by automated fluorescence microscopy with a Leica DMR 1200 upright microscope equipped with a computer-controlled translation stage (Vashaw Scientific, Inc., Frederick, MD). Image Pro software (Media Cybernetics, Carlsbad, CA) controlled the stage and image acquisition. A 10 × 10 grid of images was captured for each PDLLA film or glass slide control. Two fluorescence images were captured at each grid position: (1) a red channel image for Texas red C_2-maleimide-stained cell bodies; and (2) a blue channel image of DAPI-stained cell nuclei. A total of 19,200 images (36.5 GB) were collected for this analysis (96 specimens * 100 positions per specimen * 2 images per position * 1.9 MB per image). The red cell body images were used for determining cell area and the blue cell nuclei images were used to determine cell number. Each image had an area of 0.347 mm^2 (10× eyepiece × 10× objective = 100× magnification) and a total area of 34.7 mm^2 was imaged on each specimen (5.6% of the surface of each PDLLA film).

The number of nuclei present in each of the blue channel images and the area of each cell in the red channel images was tabulated using a macro program written for Image Pro [19]. Approximately 106,000 cells were counted and approximately 32,000 cells were sized. For analysis of cell number data, cells/mm^2 was determined for each of the 96 specimens. Cells/mm^2 for the 6

specimens from each of the 16 treatments were averaged and plotted. Differences in cell number between the four aging times for the same *surface* and *medium* treatment were compared by ANOVA with Tukey's test for multiple comparisons ($p < 0.05$).

For cell area data, a different approach was used because the cell area distributions were found to be log-normally distributed. Thus, the cell area data for each of the 96 specimens was log-transformed to make the distributions normal before analysis. As determined by ANOVA with Tukey's test for multiple comparisons ($p < 0.05$), there was no statistical difference between the data sets from the 6 specimens from identically treated samples. Therefore, cell area data from all 6 identically treated specimens were combined to yield one data set for each of the 16 treatments. Differences in cell area between the four aging times for the same *surface* and *medium* treatment were compared by ANOVA with Tukey's test for multiple comparisons ($p < 0.05$).

2.6 Vinculin focal adhesion staining

Two PDLLA films were pre-aged in MWS for 30 min and 2 PDLLA films were pre-aged in MWS for 7 d. Incubations were performed in 150 cm^2 Petri dishes with 60 mL of medium in a CO_2 incubator. After pre-aging the films, 750,000 MC3T3-E1 cells were seeded into each dish, the cells were gently mixed and the dishes were left undisturbed for 30 min to allow cell attachment. After 30 min, all dishes were moved to the CO_2 incubator for a 6 h incubation. After 6 h, cells were prepared for vinculin focal adhesion immunostaining.

For visualization of vinculin in focal adhesions, cells were extracted in 0.5% (by volume) Triton X-100 in cold cytoskeleton buffer [50 mmol/L NaCl, 150 mmol/L sucrose, 3 mmol/L $MgCl_2$, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L PIPES buffer (1,4-iperazinediethanesulfonic acid), pH 6.8] for 10 min to permeabilize the cell membranes and stabilize cytoskeletons [20]. Extracted cells were then fixed in cold 3.7% (by volume) formaldehyde (in phosphate buffered saline), blocked in 5% (by volume) fetal bovine serum and incubated with primary antibody against vinculin (murine, monoclonal anti-vinculin, V-4505, Sigma, St. Louis, MO). Next, cells were incubated in fluorescein-isothiocyanate-labeled secondary antibody (goat-anti mouse, Molecular Probes, Eugene, OR) and counterstained with Hoechst 33342 dye (Molecular Probes, Eugene, OR) to stain DNA. Cells were imaged by epi-fluorescence microscopy (manual, not automated) and two images were captured for each field: a green channel image for FITC stained focal adhesions and a blue channel image for Hoechst stained nuclei.

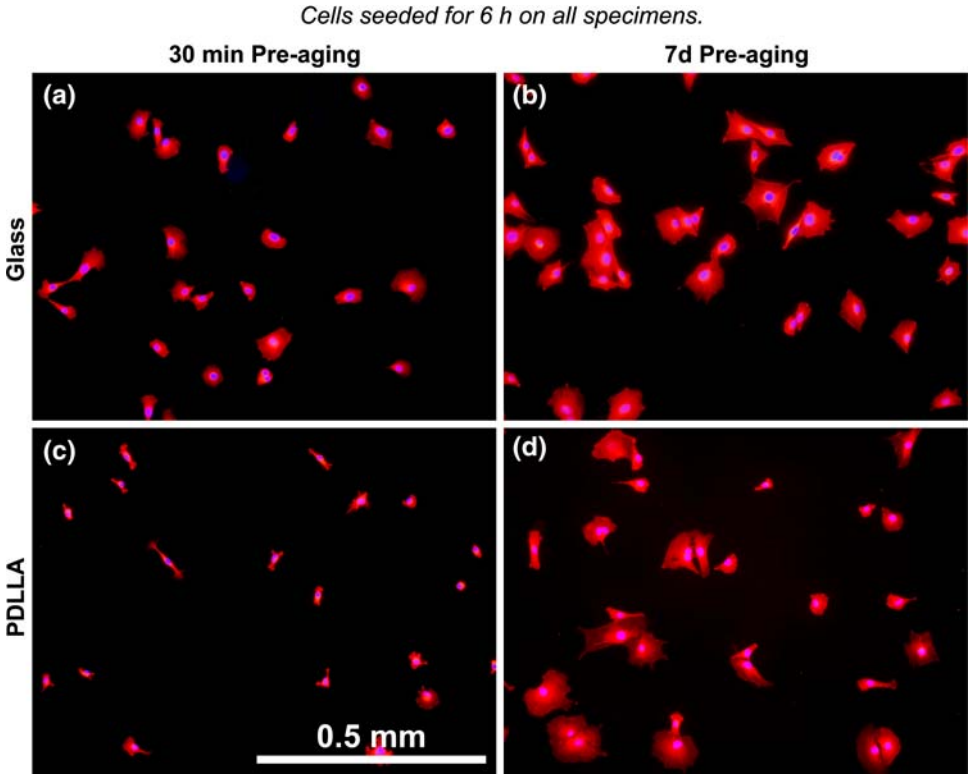

Fig. 2 Fluorescence microscopy images of cells cultured for 6 h on surfaces aged in cell medium. All surfaces in the figure were pre-aged in MWS. Panels (a) and (b) are glass slides and Panels (c) and (d) are PDLLA films. Panels (a) and (c) were pre-aged 30 min and Panels (b) and (d) were pre-aged 7 d. After pre-aging, cells were seeded on the substrates and incubated for 6 h in all cases. Cell bodies were stained red with Texas-red-C_2-maleimide and cell nuclei were stained blue with DAPI. The size bar in Panel (c) applies to all panels

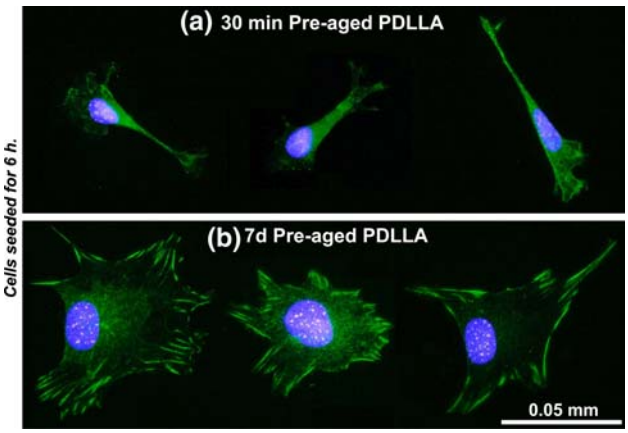

Fig. 3 Focal adhesion staining of vinculin in cells cultured on PDLLA films pre-aged in medium. PDLLA films were pre-aged in MWS for 30 min [Panel (a)] or 7 d [Panel (b)]. After pre-aging, cells were seeded on the substrates and incubated for 6 h in all cases. Immuno-histochemistry techniques were used to stain vinculin green with FITC and Hoechst was used to stain cell nuclei blue. Cells were imaged by fluorescence microscopy and the images shown are composites. The size bar in Panel (b) also applies to Panel (a)

2.7 Water contact angle and atomic force microscopy

Sixteen PDLLA films were pre-aged as follows: 4 were pre-aged 30 min in MWS, 4 were pre-aged 7 d in MWS, 4 were pre-aged 30 min in SFM and 4 were pre-aged 7 d in SFM. Incubations were performed in 150 cm^2 Petri dishes with 60 mL of medium in a CO_2 incubator. After pre-aging, films were rinsed with deionized water, blown dry with nitrogen and placed in a desiccator for 2 d. Two films from each treatment were used for water contact angle (WCA) and 2 films from each treatment were used for atomic force microscopy (AFM). For the 8 WCA films, three measurements in air were made on each film using a Krüss G2 contact angle measuring system (Matthews, NC). The water contact angle of 2 untreated PDLLA films was also determined as a control. For the 8 AFM films, films were scanned by tapping-mode AFM using a Dimension 3100 Nanoscope IIIa (Veeco Instruments, Inc., Woodbury, NY). A scan rate of 0.5 Hz was used to scan two 1 μm × 1 μm areas on each film. Two untreated PDLLA films were also scanned as controls.

2.8 Notes

Certain equipment and instruments or materials are identified in the paper to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology, nor does it imply the materials are necessarily the best available for the purpose.

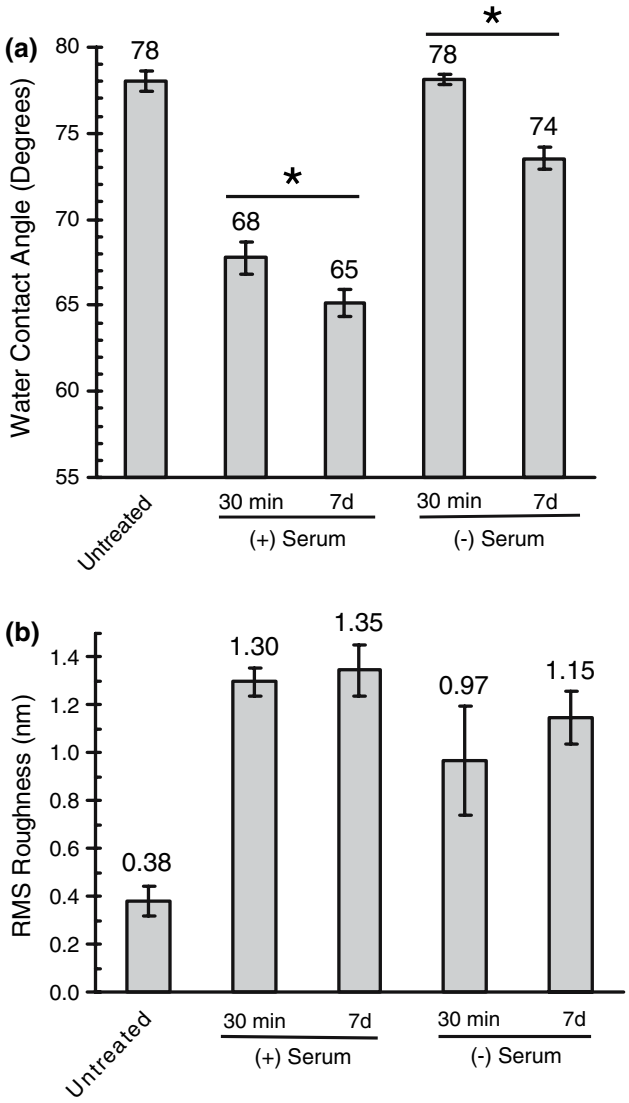

Fig. 4 Water contact angle (WCA) and surface roughness of PDLLA films pre-aged in medium. PDLLA films were pre-aged in medium with or without serum for 30 min or 7 d. After pre-aging, films were rinsed with water and stored in a desiccator for 2 d. Two untreated PDLLA films were also included as a control. (**a**) For WCA, 2 PDLLA films were pre-aged for each condition and 3 measurements were made on each film. Each bar in the figure represents the average of the 6 measurements made for each condition ($n = 6$) and error bars are standard deviation of the mean. Average values are given above each bar. *T*-tests showed that "30 min, (+) Serum" was significantly different from "7 d, (+) Serum" and that "30 min, (−) Serum" was significantly different from "7 d, (+) Serum" ($P < 0.05$). (**b**) For roughness, 2 PDLLA films were pre-aged for each condition and scanned by atomic force microscopy (AFM) (1 μm × 1 μm area). Two positions were scanned on each film and root mean square (RMS) roughness was determined from height images. Each bar in the figure represents the average of the 4 measurements made for each condition ($n = 4$) and error bars are standard deviation of the mean. *T*-tests showed no significant difference between "30 min, (+) Serum" and "7 d, (+) Serum" or between "30 min, (−) Serum" and "7 d, (+) Serum" ($p > 0.05$)

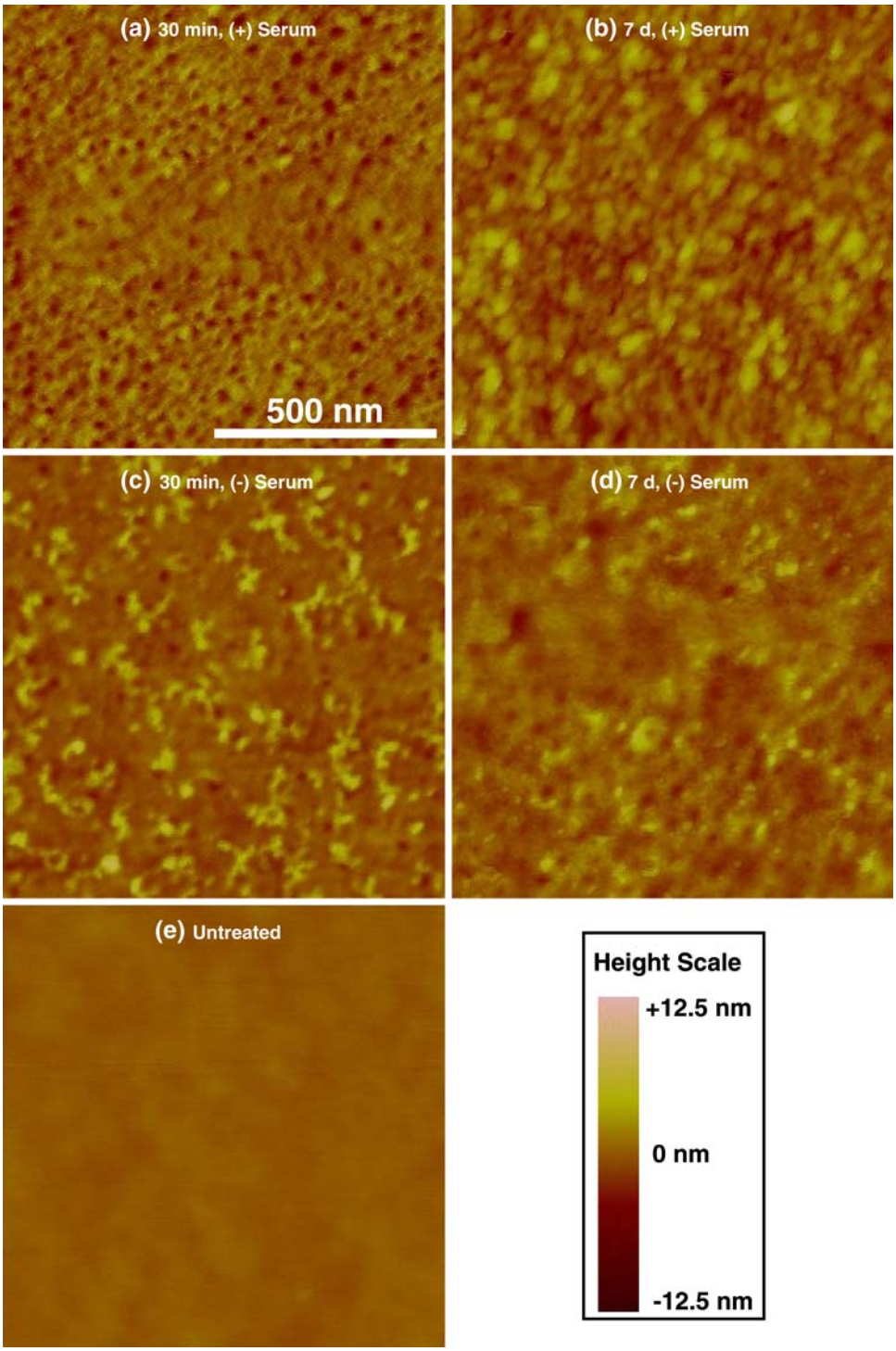

Fig. 5 Atomic force micrographs (AFM) of PDLLA films pre-aged in cell medium. PDLLA films were pre-aged in medium with or without serum for 30 min or 7 d as indicated in each panel. After pre-aging, films were rinsed with water and stored in a desiccator for 2 d before AFM. An untreated PDLLA film is shown as a control. The size bar in Panel (a) also applies to Panels (b)–(e). All images are height images and a scale is given to right of Panel (e)

3 Results & discussion

When cells were cultured for 6 h on PDLLA films that had been pre-aged in MWS for varied lengths of time, cell adhesion and spreading were enhanced on the films that had been pre-aged the longest (Figs. 1 and 2). For control glass slides, only cell spreading (not cell adhesion) was enhanced by increased pre-aging time in MWS (Figs. 1 and 2). The surface properties of glass differ from PDLLA, but glass was used as a control because it does not degrade, absorb water or swell. When SFM was used, cell adhesion was not affected by the pre-aging time of PDLLA films or glass slides, and cell spreading was reduced on PDLLA films as pre-aging time was increased (Figs. 1 and 2). Cell spreading on glass slides pre-aged in SFM was somewhat affected by pre-aging time, although there was no trend in

the data. These results demonstrate that cell adhesion and spreading on PDLLA films or glass slides can be affected by the length of time that the substrates have been incubated in cell medium.

Formation of focal adhesions during cell adhesion correlates with increased cell adhesion strength [21]. Thus, we examined whether substrate pre-aging time affected the formation of focal adhesions in cells cultured for 6 h on the pre-aged substrates. Focal adhesion formation was examined during cell adhesion to PDLLA films that had been pre-aged in MWS for 30 min or 7 d (Fig. 3). Focal adhesions were visualized by immunostaining for vinculin. Punctate vinculin staining along the periphery of the cells was observed during adhesion to PDLLA films that were pre-aged in MWS for 7d (Fig. 3b). Diffuse vinculin staining in the cytoplasm was observed for cells adherent to PDLLA films that were pre-aged in MWS for 30 min (Fig. 3a). Thus, cells assembled well-defined focal adhesions during culture on the 7 d-MWS-films but not on the 30 min-MWS-films. These results indicate the formation of focal adhesions in cells adhering to pre-aged PDLLA is influenced by the length of time that the PDLLA is pre-aged in cell medium.

In order to investigate the mechanism of the observed effects of PDLLA pre-aging time on cell adhesion and spreading, WCA (Fig. 4a) and AFM (Figs. 4b and 5) materials characterization experiments were conducted. The hydrophobicity of PDLLA films (decrease in WCA) significantly increased with increasing length of exposure to cell medium (Fig. 4a). Pre-aging time also affected the surface morphology of the PDLLA films (Fig. 5), although significant changes in surface roughness were not detected (Fig. 4b). The globular features on films pre-aged in MWS are possibly adsorbed serum proteins (Fig. 4a, b). The globules are 30–60 nm in diameter and 12–22 nm in height, similar in dimensions to previous observations of adsorbed proteins [22, 23]. The morphology of the globules changed with time of PDLLA exposure to MWS (Fig. 4a, b), which may reflect changes in adsorbed proteins (amount, composition, conformation). PDLLA films pre-aged in SFM also developed surface features (Fig. 4c, d) although morphology was different from PDLLA exposed to MWS. These changes in surface morphology of PDLLA exposed to SFM may result from surface rearrangement of polymer chains [18, 24]. These materials characterization results indicate that the hydrophobicity and surface morphology of PDLLA films are affected by pre-aging time and changes in hydrophobicity and surface morphology can affect osteoblast adhesion and spreading [19, 25, 26]. Thus, changes in PDLLA film properties (hydrophobicity and surface morphology) may be responsible for the dynamic effects of PDLLA pre-aging time on cell adhesion and spreading.

4 Conclusions

Taken together, the data show that cell adhesion, spreading and focal adhesion formation were affected by the pre-aging time of PDLLA films in cell culture medium. Materials characterization studies showed that the hydrophobicity and surface morphology of the PDLLA films changed with time of exposure to medium. These changes in PDLLA film properties (hydrophobicity and surface morphology) may be responsible for the effects of PDLLA pre-aging time on cell adhesion and spreading. The current work is the only study (to our knowledge) that demonstrates the dynamic effects of polymer exposure time to cell medium on cell adhesion and spreading. This may seem like an obvious conclusion, but this hypothesis has not been tested (to our knowledge). This result is important because it suggests that cell response to an implanted material will change as the time of material implantation increases. In summary, these results demonstrate that cell response to a degradable, biomedical polymer is dependent on the length of time that polymer has been exposed to physiological medium.

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