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Biomaterials 24 (2003) 4655–4661
Biomaterials
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Enhancement of the growth of human endothelial cells by surface roughness at nanometer scale

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Received 17 December 2002; accepted 13 May 2003

Abstract

This study investigated whether a nanometer scale of surface roughness could improve the adhesion and growth of human endothelial cells on a biomaterial surface. Different molecular weights or chain lengths of polyethylene glycol (PEG) were mixed and then grafted to a polyurethane (PU) surface, a model smooth surface, to form a nanometer (nm) scale of roughness for PU-PEG surfaces (PU-PEG_{mix}) while PEG with a molecular weight of 2000 was also grafted to PU to form PU-PEG_{2000} for comparison. In addition, the concept was tested on cell-adhesive peptide Gly–Arg–Gly–Asp (GRGD) that was photochemically grafted to PU-PEG_{mix} and PU-PEG_{2000} surfaces (e.g., PU-PEG_{mix}-GRGD and PU-PEG_{2000}-GRGD surfaces, respectively). To prepare GRGD-grafted PU-PEG_{mix} and PU-PEG_{2000} surface, 0.025 M of GRGD-SANPAH (*N*-Succinimidyl-6-[4′-azido-2′-nitrophenylamino]-hexanoate) solutions was grafted to PU-PEG_{mix} and PU-PEG_{2000} by surface adsorption of the peptide and subsequent ultraviolet (UV) irradiation for photoreaction. The grafting efficiencies for GRGD to PU-PEG_{mix} and PU-PEG_{2000} surfaces were about 67% for both surfaces, semi-quantitatively analyzed by an HPLC. The surface roughness, presented with a roughness parameter, R_a, and the topography of the tested surfaces were both measured and imaged by an atomic force microscope (AFM). Among the R_a values of the films, PU was the smoothest (e.g., $R_a = 1.53 \pm 0.20$ nm, $n = 3$) while PU-PEG_{mix} was the roughest (e.g., $R_a = 39.79 \pm 10.48$ nm, $n = 4$). Moreover, R_a values for PU-PEG_{mix} and PU-PEG_{mix}-GRGD surfaces were about 20 nm larger than those for PU-PEG_{2000} and PU-PEG_{2000}-GRGD, respectively, which were consistent with the topographies of the films. Human umbilical vein endothelial cells (HUVECs) were adhered and grown on the tested surfaces after 36 h of incubation. Among the films, HUVEC's adhesion on the surface of PU-PEG_{mix}-GRGD was the densest while that on the surface of PU-PEG_{2000} was the sparsest. Also, the adhesion and growth of HUVECs for the roughness surfaces were statistically significantly better than that of smooth surface for both GRGD grafted and un-grafted surfaces, respectively. The viability for the growth of HUVECs on the tested surfaces analyzed by MTT assay also confirmed the efficacy of the increased surface roughness.

In conclusion, increased surface roughness of biomaterial surfaces even at $10–10^2$ nm scale could enhance the adhesion and growth of HUVECs on roughness surfaces that could be useful for applications of tissue engineering.

Keywords: Surface roughness; nm scale; AFM; HUVECs; GRGD

1. Introduction

Surface-induced thrombosis is one of the major drawbacks that hampers the successful applications of some biomaterials such as polyurethane (PU) and chitosan in blood-contacting artificial medical devices. To provide a bioactive and biological–graft interface, in vitro endothelization on grafted surfaces such as polyurethane (PU) has given promising results in animal tests to improve their blood compatibility [1–3]. Various methods have been developed to support the seeding and growth of endothelial cells (HUVECs) on PU or other biomaterial surfaces such as surface modifications by plasma treatment and photochemically grafted GRGD peptide on a modified PU surface or chitosan surface by this group [4–6].

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0142-9612/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0142-9612(03)00361-2

The above-mentioned surface modifications may be categorized as chemical or biological surface modifications to enhance endothelization on biomaterial surfaces. On the other hand, modifications of physical properties of surfaces such as enhancing hydrophilic properties of surface, changing porosity of materials and increasing roughness of surface [7–9] may also enhance endothelization of biomaterials.

With regard to roughness of the surface affecting the growth of different kinds of cells, some researchers reported that increased surface roughness by coarse sand-blasted could affect cell number and production of growth factors of osteoblast-like MG-63 cells on the titanium surface [9,10]. Lampin et al. reported that increased roughness of the PMMA (polymethylmethacylate) surface by sandblasting PMMA with aluminum grain sizes of 50–150 μm could enhance cell adhesion and migration [11]. Here, we increased the roughness of the surface in nanometer scale by grafting different molecular weight/different lengths of PEG (polyethylene glycol) (e.g., PEG 1100, 2000 and 5000), a spacer, to the PU surface, a model biomaterial, namely PU-PEG_{mix}. To investigate whether increasing the roughness of nanometer scale by random molecule distribution instead of microscale of roughness by mechanical sandblasting would affect cell adhesion and growth, we grew human endothelial cells (HUVECs) on different roughness of PU-PEG surfaces. To further investigate the possible role of nanoscale roughness on biological modification or peptide-grafted surfaces, such as Arg–Gly–Asp (RGD), a cell adhesion tri-peptide, the adhesion and growth of HUVECs was also studied on different roughness of GRGD-grafted PU-PEG surfaces.

To graft GRGD on different roughness of PU-PEG surfaces, photochemical techniques based on phenyl azido chemistry have been applied. The technique has been applied to graft heparin or RGD-peptide to different biomaterial surfaces such as PU or polyvinyl alcohol (PVA), respectively [5,6,12]. In general, heparin or RGD-peptides was firstly attached to water-soluble functional moiety to form phenyl azido-derivatized polymers or proteins, and then they were grafted to material substrates by UV irradiation [5–6,12]. Here, we applied a similar technique to graft Gly–Arg–Gly–Asp (GRGD) peptide on the PU-PEG_{mix} surface by inducing photochemical reactions between the azido group and hydroxyl group of the PEG molecules as earlier report by this group [5]. Here, we characterized the roughness of the PU–PEG_{mix} and GRGD-grafted PU-PEG_{mix} surfaces (PU-PEG_{mix}-GRGD) by an atomic force microscopy (AFM) while providing the images of the surfaces. In addition, we characterize the adhesion and growth of HUVECs on the surfaces by providing the morphology and viability of cells to evaluate the roles of roughness of surfaces on cell behaviors.

2. Material and methods

2.1. Preparing PU-PEG_{2000}, PU-PEG_{mix}, surfaces

The procedures for preparing PU-PEG with PEG molecular weight (MW) of 2000 (PU-PEG_{2000}) were the same as our earlier reports [5,13]. To prepare the PU-PEG_{mix} surface, different MW/lengths of PEG molecules (i.e., PEG 1100, 2000 and 5000), purchased from Fluka Co., with a molar ratio of 1:2:1 were well mixed, and grafted onto PU surfaces [13] to form a PU-PEG_{mix} surface.

2.2. Preparing PU-PEG_{2000}-GRGD and PU-PEG_{mix}-GRGD surfaces

To further prepare GRGD-grafted PU-PEG_{2000} and PU-PEG_{mix} surfaces, the photochemical technique was applied as given in our earlier reports [5,6]. In general, GRGD (MW 403.4 g) and SANPAH (MW 492.4 g) were purchased from Pierce Chemical Corp. (Rockford, IL, USA). To graft GRGD-SANPAH (*N*-Succinimidyl-6-[4′-azido-2′-nitrophenylamino]-hexanoate) on the surface of PU-PEG surfaces, 0.025 M of GRGD and SANPAH were firstly dissolved in distilled water and pure ethanol, respectively. Then, equivalent moles of above-mentioned GRGD and SANPAH solutions were gently mixed and reacted in a dark room at room temperature for 2 h to form phenyl azido-derivatized peptides. The ethanol containing GRGD-SANPAH solution was poured into the above-mentioned PU-PEG_{2000} and PU-PEG_{mix} films. After the films were air-dried, they were irradiated by ultraviolet light (290–370 nm) for 4 min to induce photochemical fixation of GRGD on the PU-PEG_{mix} surfaces by a UV generator (Model 68805, ORIEL Instrument, Stratford, CT, USA). The film was fully rinsed with distilled water to removed un-reacted reagents and then dried at room temperature. For a semi-quantitative analysis of the grafting efficiency of GRGD-SANPAH to chitosan films, the above-mentioned distilled water, used for the washing of the samples, was collected, and further analyzed by an HPLC (Jasco PU-1580, Kobe, Japan) equipped with a C_{18} reverse phase column (#201SP54, 4.6 mm × 25 cm ID, VyDAC Corp., Hesperia, CA, USA) at room temperature. For this analysis, 10% acetonitrile dissolved in 0.1% of trifluoroacetic acid (TFA) solution was used as the mobile phase with a 0.8 ml/min flow rate, and the quantity of GRGD-SANPAH was detected by absorption intensity of wavelength at 254 nm that was analyzed with a built-in standard software of the HPLC.

2.3. Surface characterization

Contact angles for PU, PU-PEG_{2000}, PU-PEG_{5000}, PU-PEG_{1100} and PU-PEG_{mix} films were measured by a

contact angle meter (FACE CA-D, Kyowa Interface Science, G-Yu, Japan). The infrared spectra of the surfaces were detected by a Fourier transform infrared spectrum (FTIR) analyzer, collected at 4-cm^{-1} resolutions, and analyzed with built-in standard software package (Perkin-Elmer Spectrum One, Perkin-Elmer Co., Norwalk, CT, USA).

To determine different roughness of the surfaces, an atomic force microscopy (AFM) (Hitachi DI-5000, Hitachi Koki Co. Ltd, Japan) was applied to scan five different areas of surface for each sample with an area and height of 4 μm^2 and 500 nm, respectively, and then to take the images of the surface in a tapping mode. Some of the PU-PEG_{mix} surfaces were scanned with a large area (25 μm^2) for validation of the roughness measurements. To describe the roughness of the surface of the films, the topography of the surface and the roughness parameter for the surface, R_a, which is the centerline average or the distance between the highest and the lowest point of the surface irregularities, were shown and calculated by built-in software (Nanoscope IIIa, Digital Instrument, CA, USA). R_a has also been applied to describe the roughness of the surface by another group [11].

2.4. Cell culture

The cell culture procedures were the same as our earlier reports [5–6]. In general, the GRGD grafted or un-grafted different roughness PU-PEG films were cut, sterilized with 70% alcohol and dipped in HEPES (*n*-2-hydroxyl-ethylpiperazine-*n*′-2-ethane sulfonic acid) buffer for further sterilization with UV light for 2 days. After the films were further rinsed with sterilized HEPES buffer, they were placed on the bottom of a 24-well polystyrene tissue culture plate (Falcon, USA) covered with a sterilized Teflon ring to prevent floating.

The cryopreserved human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics Inc. (Lot #: 9C1020, Portland, OR, USA). To obtain the second cycle of HUVEC, a vial of cryopreserved HUVEC purchased from the above company was first de-frozen in a 37°C water bath. The number of cells in the vial was counted by a hematocytometer, and the cells were then diluted to a concentration of 1.25×10^4 viable cells/ml to 25 cm^2 of cell culture flasks (Costar, San Diego, USA) that containing medium-200 (Cascade Biologics Inc., Portland, OR, USA) supplemented with 20% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF) and 1% of antibiotic (Gibco BRL Co., Rockville, MD, USA) for subculture, and the following cell culture experiments [6]. To study cell adhesion and growth on the modified and un-modified chitosan surfaces, 1 ml of EC suspensions harvested from three to four cycles of subculture with the above-mentioned cell density was taken and seeded onto a 24-well polystyrene tissue culture plate covered with PU-PEG_{2000}, PU-PEG_{mix}, PU-PEG_{2000}-GRGD and PU-PEG_{mix}-GRGD, respectively. The EC culture wells were incubated at 37°C with 5% CO_2/95% of air and at approximately 90% relative humidity for 36 h.

After 36 h of incubation, the cells adhering to the films were washed with phosphate-buffered saline (PBS), then incubated with 75% alcohol at 4°C for 1 h. After the samples were washed with PBS, propidium iodine (PI) (Sigma Chemicals, St. Louis, MO, USA), a dye for fluorescence stain for nucleic acids of cells, was added to stain the cells for morphological observation [6]. The morphology of the cells on the films was observed by a phase contrast microscope equipped with a fluorescence light source (Nikon TE-100, Tokyo, Japan), and photographs were taken with a CCD camera. In addition, the viability of the cells was determined by thiazolyl blue assay (MTT reagent, Sigma Chemicals, St. Louis, MO, USA) with minor modification of Mosmann method [14]. 300 μl MTT solution was firstly incubated with the cells in wells of culture plates, two types of PU-PEG and PU-PEG-GRGD films at 37°C for 4 h and then dimethyl sulfoxide solution (DMSO, Sigma Chemicals, St. Louis, MO, USA) was added to dissolve formazan crystals. The absorbance of formazan solutions obtained from the above-mentioned films was measured by an ELISA microplate reader at 570 nm (EL × 800, Bio-Tek Instruments, Inc., Winooski, Vermont, USA) [14]. For comparison, the absorbance of formazan solution measured from polystyrene cell culture well (PS) was assigned as a control group. The ratio of the differences in absorbance of the formazan solutions between the groups of PU-PEG_{mix} or GRGD-grafted PU-PEG_{mix} film and the PS control to that of the PS control group was defined as the relative cell growth rate. All calculations were analyzed by Sigmastat statistical software (Jandel Science Corp., San Rafael, CA, USA). Statistical significance was evaluated at 95% of confidence level or better. Data presented are mean $\pm$s.d.

3. Results and discussion

The schematic graphs for different roughness of PU-PEG_{2000}, PU-PEG_{mix} and PU-PEG_{mix}-GRGD surfaces are shown (Figs. 1a–c). The contact angles for the films are also shown in Table 1. The contact angle values for PU and PU-PEG_{2000} were about the same as our earlier report [5]. In addition, the contact angles for PU-PEG surfaces increased with increasing molecule weight/chain length of PEG, which was similar to others [15,16]. Since the PU-PEG_{mix} surface was prepared by mixing three different chain lengths of PEG molecules, the mean value of the contact angle for PU-PEG $_{mix}$, which was within the maximum and minimum measurement range,

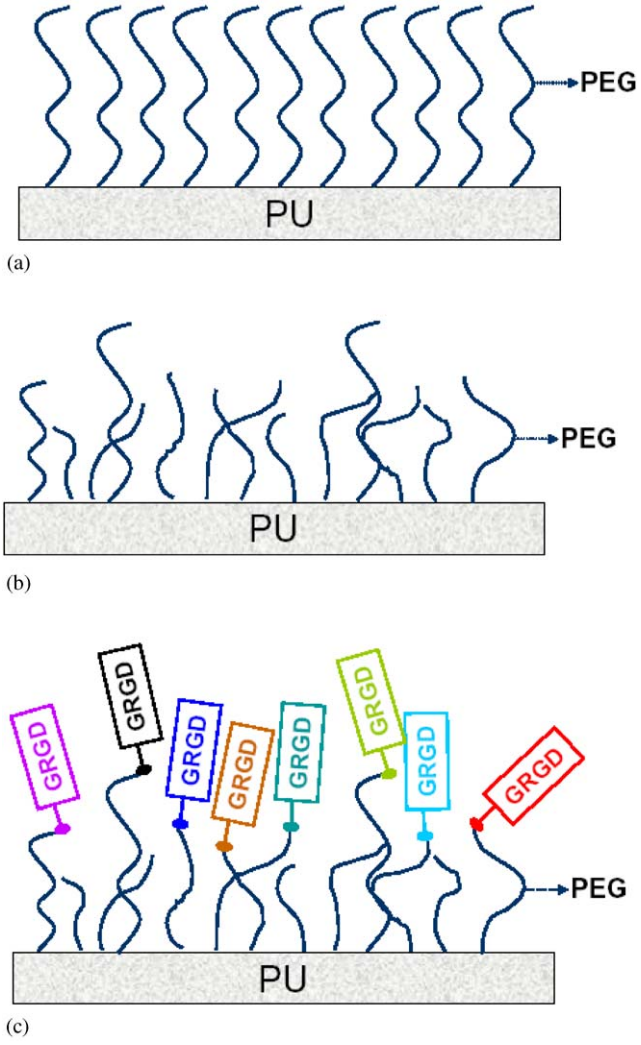

Fig. 1. The schematic diagrams for different roughness of surfaces using PU as a model biomaterial: (a) PU-PEG_{2000}, (b) PU-PEG_{mix} and (c) PU-PEG_{mix}-GRGD.

Table 1
Contact angles for PU, PU-PEG $_{5000}$, PU-PEG_{2000}, PU-PEG_{1100}, PU-PEG_{mix} films (data presented are mean $\pm$SD, $n = 8$)

Materials	PU	PU-PEG_{5000}	PU-PEG_{2000}	PU-PEG_{1100}	PU-PEG_{1100}
Angle (deg)	78.0±1.9	32.4±1.9	23.6±1.7	20.6±2.0	26.5±4.7

with a large standard deviation was reasonable. The contact angles for PU-PEG_{2000}-GRGD, PU-PEG_{mix}-GRGD would be similar to those for PU-PEG_{2000} and PU-PEG_{mix} as our earlier report [5].

The ATR-FTIR spectroscopy was performed for the films to characterize GRGD grafted on the PU-PEG_{mix} surface. The results of these functional groups such as the carboxyl group of GRGD grafted on PU-PEG surfaces were confirmed and similar to our earlier reports [5,6] (figure not shown). For example, there were absorption peaks at 963 and 1278 cm^{-1} which was attributed by carboxyl groups of CH_2–CH_2–COOH and COOH of aspartic acid. In addition, the semi-quantitative analysis of grafting efficiency of GRGD on the surfaces was carried out by analyzing the concentration of GRGD, by HPLC, of the washing solutions of UV-irradiated different roughness of PU-PEG surfaces. The concentration response peak at a retention time of 3.50 min as assigned for GRGD-SANPAH, and the intensity areas of GRGD for the washing solutions of PU-PEG_{2000}-GRGD and PU-PEG_{mix}-GRGD were much less than that of the initial grafting concentration [6]. The grafting efficiencies of GRGD-SANPAH to PU-PEG_{2000} and PU-PEG_{mix} surfaces were about 67% for both surfaces. According to the grafting efficiencies, the surface densities for GRGD grafted to two different roughnesses of PU-PEG films were about 42 nmole/cm^2.

Topographies of PU, PU-PEG_{2000}, PU-PEG_{2000}-GRGD, PU-PEG_{mix}, PU-PEG_{mix}-GRGD films were observed by AFM and shown (Fig. 2a–e) Moreover, the roughness of the films presented with R_a values that was applied to describe the roughness of surface [10] are shown (Table 2). The image of the smooth PU surface is observed (Fig. 2a) that is consistent with the R_a values for the surface (e.g., less than 2 nm). The R_a value for PU-PEG_{mix} is the largest among the tested films that indicates the most roughness of the surface (Table 2). In addition, the R_a values for PU-PEG_{mix}-GRGD and PU-PEG_{2000}-GRGD are little smaller but there is no statistical difference compared to those for PU-PEG_{mix} and PU-PEG_{2000} films, respectively. Therefore, the GRGD-grafted procedure was not effectively affected by the roughness of surfaces significantly. With regard to measurements for the roughness of the surface, the R_a value for PU-PEG_{2000} is the same order as that of the theoretically calculated chain length of PEG-2000 (e.g., extended chain length = 22.8 nm) grafted to a smooth PU surface. By means of the calculation, the experimental results hinted that the graft of PEG-2000 to the PU surface was not so uniform if micrometer (μm) scale of area was counted but it is still reasonable to assume it to be relatively smooth for a large scale, for example, centimeter scale of area for cell culture. Moreover, it is noted that the scale of roughness for PU-PEG_{mix} and PU-PEG_{mix}-GRGD are about 20 nm larger than PU-PEG_{2000} and PU-PEG_{2000}-GRGD, respectively, that is much less than the micro-scale of surface roughness fabricated by a sand-blasted technique on different surfaces by earlier groups [9–11].

The adhesion and proliferation of HUVECs on the PU-PEG_{mix} and PU-PEG $_{mix}$-GRGD films were more pronounced than that of the PU-PEG_{2000} and PU-PEG_{2000}-GRGD, respectively. Micrographs of HUVECs growth on PU-PEG_{mix} and PU-PEG_{mix}-GRGD were shown after cells were stained (Fig. 3a and b). In

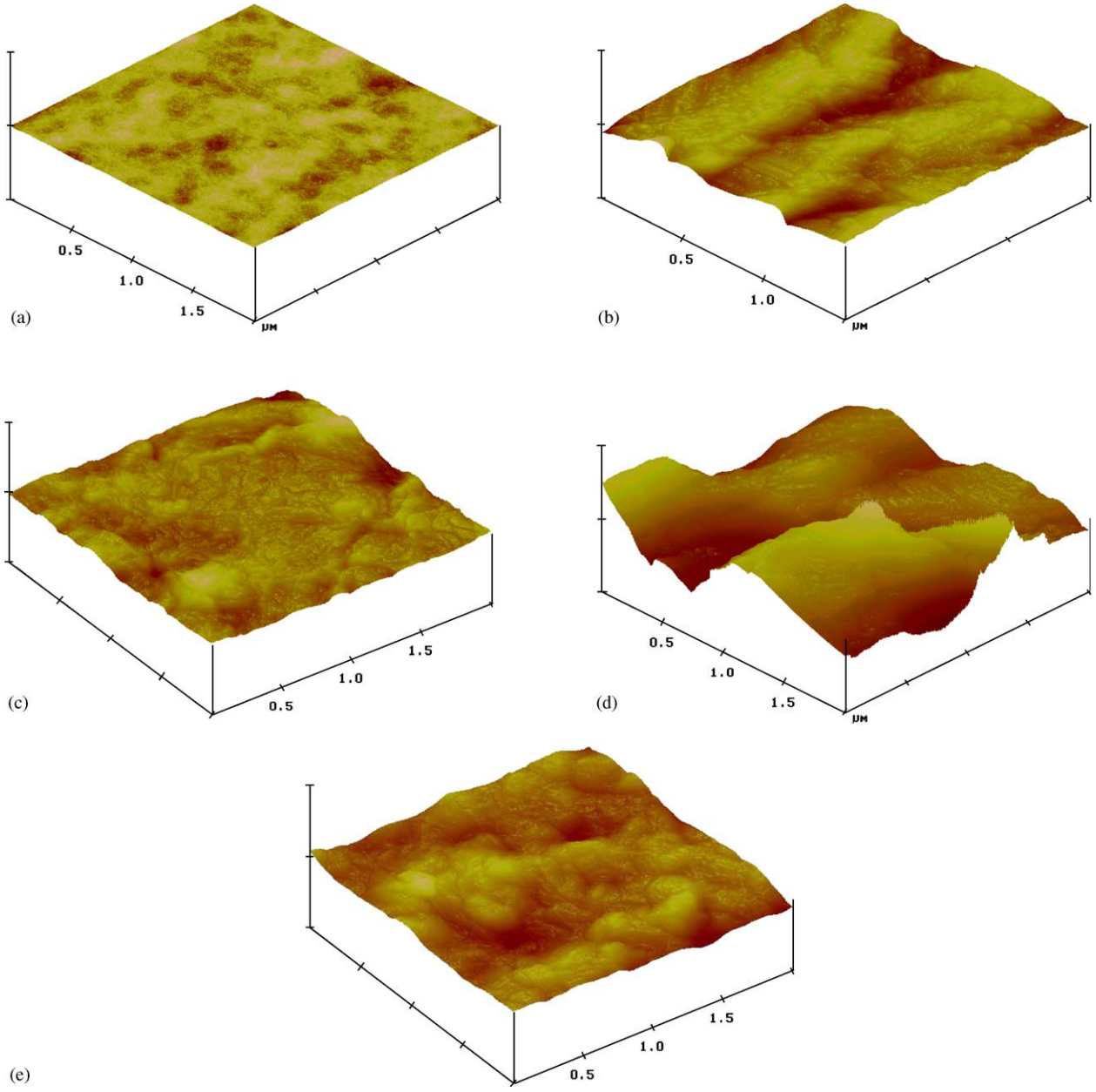

Fig. 2. Topographies of different roughness of surfaces with an area of 4 μm^2 were observed by AFM and shown: (a) PU, (b) PU-PEG_{2000}, (c) PU-PEG_{2000}-GRGD, (d) PU-PEG_{mix}, (e) PU-PEG_{mix}-GRGD films. Among the topographies, the PU surface was the smoothest while the surface of PU-PEG_{mix} was the roughest.

Table 2
Values of roughness parameter, R_a, for the surfaces obtained from AFM measurements

Materials	PU	PU-PEG_{2000}	PU-PEG^{*}_{mix}	PU-PEG_{2000}-GRGD	PU-PEG_{mix}-GRGD
Roughness R_a (nm)	1.53 ± 0.20	20.10 ± 7.87	39.79 ± 10.48	18.63 ± 5.30	34.58 ± 9.89

The R_a values showed that the PU surface was the smoothest while PU-PEG_{mix} was the roughest among the tested surfaces (data presented are mean $\pm$SD, $n = 3$; *: $n = 4$).

general, the adhered cells on the PU-PEG_{mix} and PU-PEG_{mix}-GRGD films were denser than less roughness of PU-PEG_{2000} and PU-PEG_{2000}-GRGD films, respectively. Furthermore, denser and more spread of cells of GRGD grafted surfaces than un-grafted ones were also observed as in our earlier reports [5,6]. Since MTT assay can reflect the level of cell metabolism [14], the viability for the growth rate of HUVECs determined by

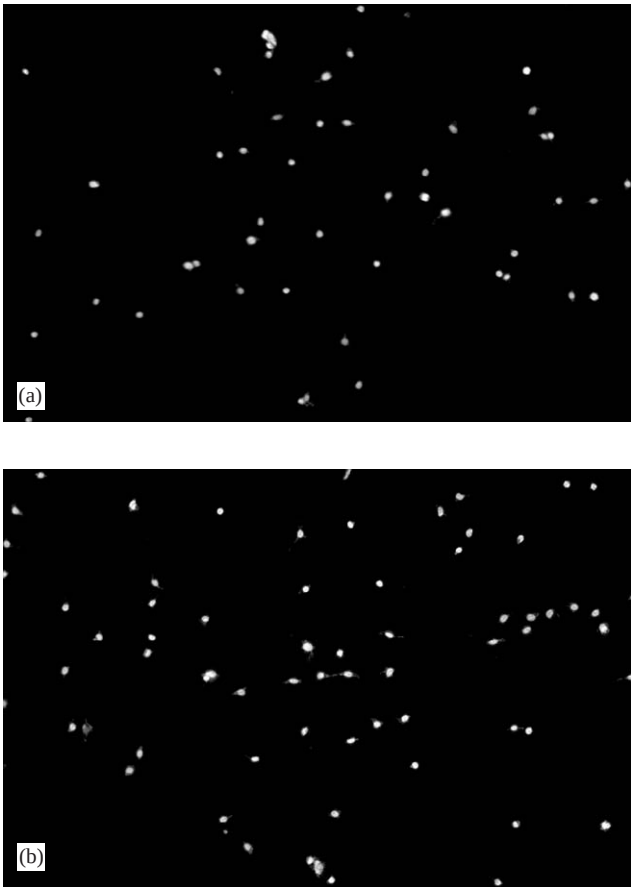

Fig. 3. (a,b) Fluoresced micrographs of HUVECs growth on: (a) PU-PEG_{mix} surface (100 ×), (b) PU-PEG_{mix}-GRGD surface (100 ×) taken after 36 h of incubation.

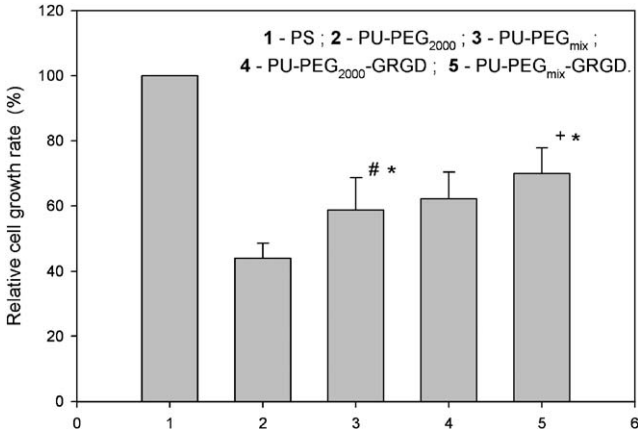

Fig. 4. Viability of HUVEC cells growth on (1) polystyrene cell culture wells (PS), (2) PU-PEG_{2000}, (3) PU-PEG_{mix}, (4) PU-PEG_{2000}-GRGD, (5) PU-PEG_{mix}-GRGD films tested by MTT assay. After processing the absorbance of formazan solution, the relative growth rates for those films are shown. (#: $P<0.001$ for groups 2 and 3; +: $P<0.04$ for groups 4 and 5; *; $P<0.005$ for groups 3 and 5; data presented are mean ± SD, $n=6$).

the assay by measuring the absorbance of the formazan solution at 570 nm has been widely applied [5–6,17–18]. Here, the results for MTT assay for viability of growth of HUVECs on the tested films are shown with the relative cell growth rates (Fig. 4). The absorbance values of the formazan solutions for cell growth on the PS well (i.e., the control group) and the different roughness of PU-PEG_{mix} and PU-PEG_{mix}-GRGD films were 0.048 ± 0.003 ($n=6$), 0.028 ± 0.003 ($n=6$) and 0.0033 ± 0.002 ($n=6$), respectively. It was also noted that GRGD grafted on PU-PEG_{mix} and PU-PEG_{2000} films enhanced cell adhesion and growth on the films compared to that on un-grafted ones ($P<0.05$ and $P<0.001$, $n=6$, respectively). Moreover, there was a significant enhancement (e.g., about 35% increases, $P<0.001$, $n=6$) for cell adhesion on PU-PEG $_{mix}$ film compared to that for the PU-PEG_{2000} film. In addition, there was also a significant enhancement for cell adhesion on PU-PEG $_{mix}$-GRGD than that of PU-PEG_{2000}-GRGD ($P<0.04$, $n=6$).

The results of enhanced cell adhesion and growth on PU-PEG_{2000}-GRGD and PU-PEG_{mix}-GRGD surfaces compared with that on GRGD un-grafted surfaces, respectively, were consistent with our earlier reports and reports of others [3,5–6,12]. It is well known that RGD tri-peptide is the minimal cell recognizable sequence for many adhesion plasma and extracellular proteins including the von Willebrand factor, fibrinectin and collagen [19]. In addition, the RGD tri-peptide plays a crucial role in mediating cell attachment and subsequently spreading [19–20]. Therefore, the enhancement of HUVECs adhesion and growth on GRGD-grafted surfaces compared with un-grafted ones in this study was reasonable.

Our results show that the effect of nano-scale of surface roughness in enhancing cell adhesion and growth on its surface are valid in both GRGD-grafted and un-grafted cases (Table 2). It indicates that the roughness of the surface, a physical factor, can alternate cell behavior such as adhesion on matrix even at the GRGD peptide (or biological) domination surface. More interestingly, the scale of roughness is only 10^1–10^2 nanometer (nm) applied in this study instead of 10^1–10^2 μm, accomplished by other groups [9–11].

By means of earlier studies, Lampin et al. reported that the effect of roughness of PMMA surfaces in enhancing cell adhesion might be due to triggering of sub-confluent cells to secrete extra-cellular proteins which allowed better anchorage of cells to their substratum [11] while others reported that the roughness of the titanium surface could modulate the product of cytokine and growth factor of cells, but reduced cell numbers [10]. Although the results for the above studies showed different effects for cell growth on the roughness surfaces [9–11], different tested material surfaces (e.g., titanium and PMMA, respectively) and types of cells (e.g., osteoblast-like MG-63 cells and chicken embryo

vascular cells, respectively) applied for their studies could be accounted. The above-mentioned factors reported by earlier reports [9–11] might play a role in enhancing the growth of HUVECs in this study although a different cell type was used compared to theirs. However, the details of the mechanisms for enhancing HUVECs' adhesion and growth by increased roughness of surface in nm scale still need to be further investigated.

By means of the study with PU as a model, we conclude that increased roughness of surface even at 10^1–10^2 nm scales can enhance HUVECs' adhesion and growth on its surface for both GRGD-grafted and ungrafted surfaces that can be further applied in tissue engineering.

Acknowledgements

This research was supported by the National Science Council of Taiwan, ROC, project # NSC-91-2320-B-224-001 and NSC-91-2320-B-002-143. We thank the valuable discussion about the concept of nano-technology with Dr. Wen-Pin Chen.

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