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Enhancement the Growth of Human Endothelial Cells by Surface Roughness at Nanometer Scale

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Abstract

This study investigated whether a nano-meter scale of surface roughness could improve the adhesion and growth of human endothelial cells on biomaterial surface. Different molecular weights or chain lengths of polyethylene glycol (PEG) were mixed and then grafted to polyurethane (PU) surface, a model smooth surface, to form a nano-meter (nm) scale of roughness for PU-PEG surfaces (PU-PEG $_{mix}$) while PEG with 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) 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). The adhesion and growth of HUVECs for the roughness surfaces were statistical significantly better than that of smooth surface for both GRGD grafted and un-grafted surfaces, respectively.

In conclusion, increased surface roughness of biomaterial surfaces even at $10\sim10^2$ nm scale could enhance the adhesion and growth of HUVECs on roughness surfaces that could be worth for applications of tissue engineering.

Keyword: surface roughness, nm scale, AFM, HUVECs**,** GRGD

Introduction

Surface induced thrombosis is one of major drawback 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. 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 modified PU surface or chitosan surface by this group [1-3].

In regard to roughness of surface in affecting the growth of different kinds of cells, some researchers reported that increased surface roughness by coarse sand-blasted could affect of cell number and production of growth factors of osteoblast-like MG-63 cells on titanium surface [4,5]. Lampin et al reported that increased roughness of PMMA (polymethylmethacylate) surface by sandblasting PMMA with aluminum grain sizes of 50 μm to 150 μm could enhance cell adhesion and migration [6]. Here, we increased the roughness of surface in nano-meter scale by grafting different molecular weight/ different lengths of PEG (polyethylene glycol) (e.g., PEG 1100, 2000 and 5000), a spacer, to PU surface, a model biomaterial, namely PU-PEG $_{mix}$. To investigate whether increasing the roughness of in nano-meter scale by random molecule distribution instead of micro-scale of roughness by mechanical sound-blasted 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 nano-scale 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.

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. Here,

we applied a similar technique to graft Gly-Arg-Gly-Asp (GRGD) peptide on PU-PEG $_{mix}$ surface by inducing photochemical reactions between azido group and hydroxyl group of the PEG molecules. 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) with 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.

Materials and Method

Preparing PU-PEG$_{2000}$, PU-PEG $_{mix}$, surfaces

The procedures for preparing PU-PEG with PEG molecular weight (M.W.) of 2000 (PU-PEG$_{2000}$) were the same as our earlier reports [2,7]. To prepare PU-PEG $_{mix}$ surface, different M.W./ lengths of PEG molecules (i.e., PEG 1100, 2000 and 5000), purchased from Fluka Co., with molar ratio of 1:2:1 were well mixed, and grafted to PU surfaces [7] to form a PU-PEG $_{mix}$ surface.

Preparing PU-PEG $_{2000}$ -GRGD and PU-PEG $_{mix}$-GRGD surfaces

To further prepare GRGD grafted PU-PEG$_{2000}$ and PU-PEG $_{mix}$ surfaces, photochemical technique was applied. In general, GRGD (Mw.403.4g) and SANPAH (Mw. 492.4g) 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 abovementioned GRGD and SANPAH solutions were gently mixed and reacted in a dark room at room temperature for two hours 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 minutes 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 a HPLC (Jasco PU-1580, Kobe, Japan) equipped with a C_{18} reverse phase column (#201SP54, 4.6mmx25cm ID, VyDAC Corp., Hesperia, CA, USA) at room temperature.

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, 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 area and height of 4 μm^2 and 500 nm, respectively and then to take the images of the surface in a tapping mode. 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 surface by another group [6].

Cell culture

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-ethanesulfonic 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 wells polystyrene tissue culture plate (Falcon,

USA) covered with a sterilized Tefelon 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 firstly de-frozen in a 37 ^{0}C of water bath. The number of the cells in the vial was counted by a hematocytometer, and the cells were then diluted to a concentration of 1.25 x10^4 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 [3]. 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^{0}C with 5% CO_2 / 95 % of air and at approximately 90% relative humidity for 36 hrs.

After 36 hrs of incubation, the cells adhering to the films were washed with phosphate buffered saline (PBS), then incubated with 75% alcohol at 4 ^{0}C for 1 hr. After the samples 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 [3]. The morphology of the cells on the films was observed by a phase contrast microscope equipped with 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 ^{0}C for 4 hrs 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 (ELx800, Bio-Tek Instruments, Inc., Winooski, Vermont, USA) [8]. For comparison, the absorbance of formazan solution measured from polystyrene cell culture well (PS) was assigned as a control group. 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 ± s.d.

Results and Discussion

The schematic graphs for different roughness of PU-PEG$_{2000}$, PU-PEG $_{mix}$ and PU-PEG $_{mix}$ -GRGD surfaces are shown (Fig.1a-c). The contact angles for the films are also shown in Table I. Since PU-PEG $_{mix}$ surface was prepared by mixing three different chain lengths of PEG molecules, the mean value of contact angle for PU-PEG $_{mix}$, which was within the maximum and minimum measurement range, with a large standard deviation was reasonable.

The ATR-FTIR spectroscopy was performed for the films to characterize GRGD grafted on PU-PEG $_{mix}$ surface. The results of those functional groups such as carboxyl group of GRGD grafted on PU-PEG surfaces were confirmed For example, there were absorption peak 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 the UV irradiated different roughness of PU-PEG surfaces. The concentration response peak at a retention time of 3.50 minutes was 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. 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.2.a-e) Moreover, the roughness of the films presented with R_a values that was applied to describe the roughness of surface are shown (Table II). The image of 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 II). In addition, the R_a values for PU-PEG $_{mix}$-GRGD and PU-PEG$_{2000}$-GRGD are little smaller but 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 the roughness of surfaces in significance. Regards to measurements for the roughness of 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 smooth PU surface. Through the calculation, the experimental results hinted that the grafted of PEG-2000 to PU surface was not so uniform if micrometer (μm) scale of area was counted but it is still reasonable to assume relative 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 micro-scale of surface roughness fabricated by sandblasted technique on different surfaces.

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 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. Since MTT assay can reflect the level of cell metabolism, the viability for the growth rate of HUVECs determined by the assay with measuring the absorbance of the formazan solution at 570 nm has been widely applied. 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 PU-PEG$_{2000}$ film. In addition, there was also 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 others. The RGD tri-peptide plays a crucial role in mediating cell attachment and subsequently spreading. 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 on enhancing cell adhesion and growth on its surface are valid on both GRGD grafted and un-grafted cases (Table II). It indicates that the roughness of surface, a physical factor, can alternate cell behavior such as adhesion on matrix even at GRGD peptide (or biological) domination surface.

Through the study with PU as a model, we conclude that increased the 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 un-grafted surfaces that can be further applied in tissue engineering

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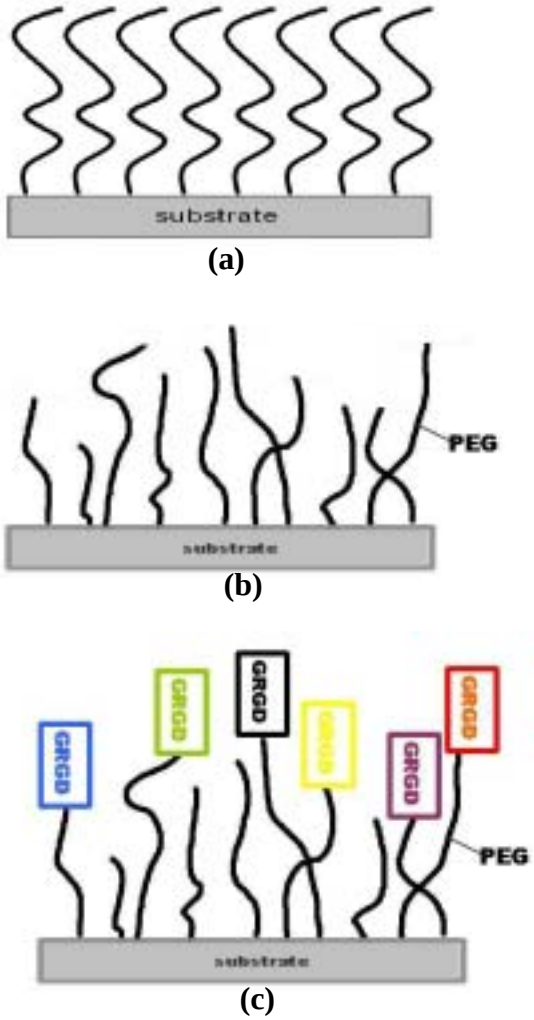

Fig. 1. The schematic diagrams for different roughness of surfaces by using PU as a model biomaterial: a). PU-PEG $_{2000}$, b). PU-PEG $_{mix}$ and c). PU-PEG $_{mix}$ -GRGD.

Table I. Contact angles for PU, PU-PEG $_{5000}$, PU-PEG$_{2000}$, PU-PEG $_{1100}$, PU- PEG $_{mix}$ films. (Data presented are mean ± SD, n=8)

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

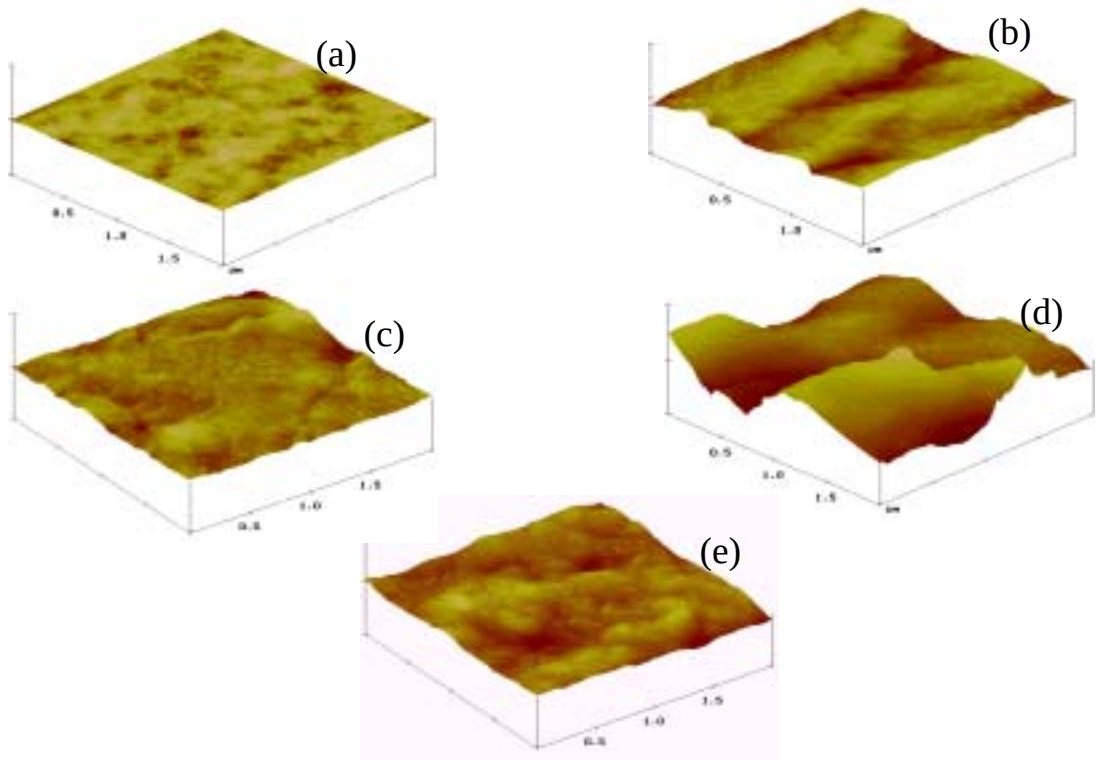

Fig. 2. Topographies of different roughness of surfaces with area of 4 μm^2 were observed by AFM and shown: a). PU, b). PU-PEG_{2000}, c). PU-PEG_{2000}-GRGD, d). PU-PEG_{mix}, e). PU-PEG_{mix} - GRGD films. Among the topographies, PU surface was the smoothest while the surface of PU-PEG_{mix} was the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

Table II. The values of roughness parameter, R_a, for the surfaces obtained from AFM measurements. The R_a values showed that PU surface was the smoothest while PU-PEG_{mix} was the roughest among the tested surfaces. (Data presented are mean ± SD, n=3; * : n=4).

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

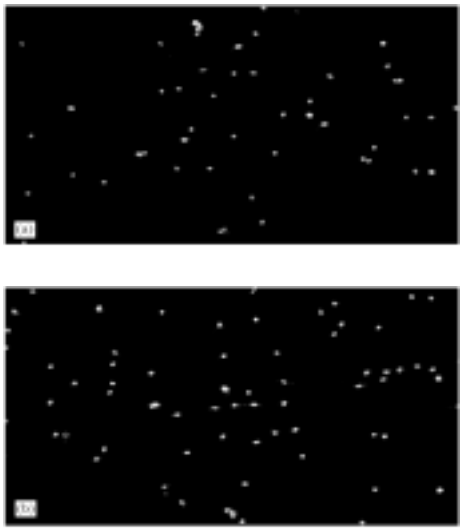

Fig. 3a and 3b. Fluoresced micrographs of HUVECs growth on: a). PU-PEG_{mix} surface (100 x), b). PU-PEG_{mix} - GRGD surface(100 x) taken after 36 hrs of incubation.

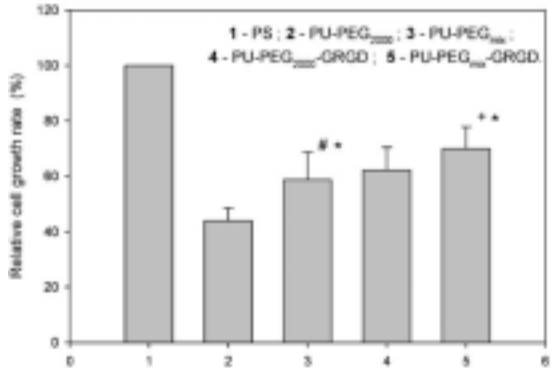

Fig.4. Viability of HUVEC cells growth on 1. Polystyrene cell culture wells (PS), 2.PU- PEG_{2000}, 3. PU- PEG_{mix} , 4. PU-PEG_{2000}-GRGD, 5. PU-PEG_{mix} - GRGD films tested by MTT assay. After processing the absorbance of formazan solution, the relative growth rates for those films are shown. (#: P<0.001 for group2 and 3; +: P<0.04 for group 4 and 5; *; P< 0.005 for group 3 and 5; data presented are mean ± SD, n=6).