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Fabrication of PHBV/Keratin Composite Nanofibrous Mats for Biomedical Applications

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Abstract: Keratin is an important protein used in wound healing and tissue recovery. In this study, keratin was modified chemically with iodoacetic acid (IAA) to enhance its solubility in organic solvent. Poly(hydroxybutylate-*co*-hydroxyvalerate) (PHBV) and modified keratin were dissolved in hexafluoroisopropanol (HFIP) and electrospun to produce nanofibrous mats. The resulting mats were surface-characterized by ATR-FTIR, field-emission scanning electron microscopy (FE-SEM) and electron spectroscopy for chemical analysis (ESCA). The pure *m*-keratin mat was cross-linked with glutaraldehyde vapor to make it insoluble in water. The biodegradation test *in vitro* showed that the mats could be biodegraded by PHB depolymerase and trypsin aqueous solution. The results of the cell adhesion experiment showed that the NIH 3T3 cells adhered more to the PHBV/*m*-keratin nanofibrous mats than the PHBV film. The BrdU assay showed that the keratin and PHBV/*m*-keratin nanofibrous mats could accelerate the proliferation of fibroblast cells compared to the PHBV nanofibrous mats.

Keywords: biomedical, fibrobalsts, keratin, nanofiber, PHBV.

Introduction

Keratin is a chief component found in hair, skin, fur, wool, horns, and feathers. Reinforced with calcium salts, it is also found in hooves, nails, claws and beaks.[1] Keratin can be used in a variety of biomedical applications due to its biocompatibility and biodegradability. Film made of keratin can be used as wound dressing,[2] tissue engineering scaffold,[3] a coating for implantable devices,[4,5] and cell encapsulant.[6] The three dimensional product of keratin can be used as a cross-linked implantable biomaterial for soft and hard tissue replacement. Keratin is extremely insoluble, so the primary task is to enhance it's solubility in water or organic solvent. Various attempts have been made to obtain water soluble keratin with 2-mercaptonethanol or mercaptoacetic acid after the reduction of disulphide bonds.[7,8] Yamauchi *et al.* has extracted keratin from wool and explored its interaction with cell.[9,10] Schrooyen *et al.* also extracted keratin from feathers, and partially modified it.[11,12]

Electrospinning has been recognized as an efficient technique for the fabrication of polymer nanofibers. Very recently, some natural biopolymers, which include recombinant elastin,[13] silk,[14,15] fibrinogen,[16] gelatin,[17,18] chitosan,[19,20] and collagen,[21-23] have been successfully electrospun. However, keratin nanofibers have not been prepared. Compared with synthetic polymers, natural biopolymers possess good biocompatibility. However, their processability is, in general, pretty poor. The successful conversion of natural biopolymers into ultrafine and nanofibrous forms will provide new opportunity and enhance their efficacy in bioengineering applications.[18]

In this study, we chemically modified keratin by using iodoacetic acid (IAA). The protein content and modification degree of keratin were determined by the Bradford method and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) assay, respectively. The modified-keratin was blended with poly (hydroxybutylate-*co*-hydroxyvalerate) (PHBV) and electrospun to

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produce biodegradable cell adhesive mats. The resulting mats were characterized using ATR-FTIR spectroscopy (Jasco-620, Tokyo, Japan) and electron spectroscopy for chemical analysis (ESCA LAB VG microtech, Mt500/1etc, East Grin, UK). The biodegradation of the nanofibers were evaluated using PHB depolymerase and trypsin. The behavior of the fibroblasts on nanofibrous mats was also investigated.

Experimental

Materials. Keratin was obtained from the MP Biomedical Company, Germany. 3-Hydroxybutyric acid-*co*-hydroxyvaleric acid (PHBV, PHV content: 5%), iodoacetic acid (IAA), 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) and dialysis bags (MWCO 12,000-14,000) were purchased from the Aldrich Chemical Co (MO, USA).

Preparation of Modified Keratin (*m*-Keratin).[9-12] The process to prepare *m*-keratin includes two steps: extraction and chemical modification. Keratin (12 g) was mixed with urea (250 g), SDS (20 g), 2-mercaptoethanol (50 mL) and water (600 mL) in a 1,000 mL round-bottom flask. The pH of the mixed solution was adjusted to 9 using 1 M of NaOH and kept stirring for 12 h at 60 °C. The resulting mixture was filtrated through a glass filter. Subsequently, the filtrate was dialyzed against deionized water containing 0.1 wt% 2-mercaptoethanol to afford a colorless and clear solution. The dialysate was replaced every 12 h and dialysis was stopped after 48 h. The concentration of sulphydryl groups in this solution was measured using a DTNB.[24]

By treating the keratin with 2-mercaptoethanol, most of disulfide bonds in the keratin are broken and changed into sulphydryl (SH) groups. The SH groups are very easily oxidized into disulfide bonds again. Therefore, in order to increase water solubility of keratin, it is necessary to protect SH groups from oxidation. The most popular compound used for the protection of SH groups is iodoacetic acid (IAA).[25,26] The molar ratio of IAA/SH was set at 5 to give S-(carboxymethyl) keratin (Figure 1). The calculated amount of IAA was mixed with the keratin solution, and then stirred for 1 h at room temperature. After reaction, the amount of the SH group was measured again to evaluate the modification degree. The pH of the *m*-keratin solution was adjusted to 7 using 1 M of NaOH and dialyzed thoroughly for 3 days. Finally, this solution was lyophilized to obtain modified keratin (*m*-keratin).

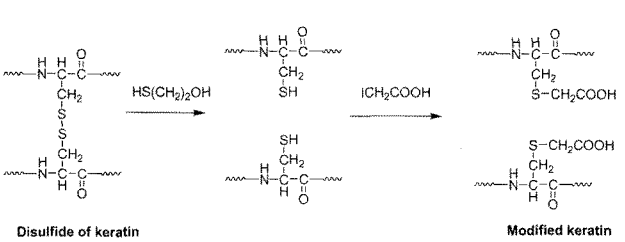

Figure 1. Formation of sulphydryl groups and their protection for enhancement of keratin solubility.

Fabrication of Nanofibrous Mats by Electrospinning. The transparent polymer solution for electrospinning was obtained by dissolving *m*-keratin and PHBV in HFIP with sufficient stirring at room temperature. In order to examine the effect of *m*-keratin content on fiber morphology, the polymer solution was prepared using the different ratios of PHBV and *m*-keratin. The blended solution was delivered to a metal needle (18 G) connected to a high voltage power supply. In this study, the typical parameters of electrospinning were as follows. The voltage was 10 KV and the distance between the spinneret and the drum was 15 cm. The feed rate was 1 mL/h for 6 wt% PHBV/*m*-keratin in HFIP.

Cross-Linking of Keratin Nanofiber Mats.[27] *m*-Keratin nanofiber mats need to be cross-linked to reduce their solubility in water. The electrospun keratin nanofibrous mats was cross-linked by treating them with glutaraldehyde vapor and saturated with a 25% glutaraldehyde aqueous solution at room temperature for various time periods. This was followed by treatment with 0.1 M glycine aqueous solution to block unreacted aldehyde groups. The crosslinked mats were dried and weighed (W_d). The initial weight of mats was W_0. The weight loss was calculated using the following equation:

$$\text{Weight loss percentage} = (W_0 - W_d)/W_0 \times 100\%$$

***In vitro* Biodegradation.** The electrospun nanofiber mats were cut into rectangles (20×20×0.05 mm) for *in vitro* degradation testing. Each specimen was placed in a test tube containing 10 mL of phosphate-buffered saline (PBS, pH 7.0, Gibco) and incubated for 12 h at 37 °C. After incubation, the samples were washed and lyophilized for 24 h. In order to measure the enzymatic degradation of nanofibrous scaffolds, the samples were incubated in PBS at 37 °C in the presence of *pseudomonas stutzeri* BM190 depolymerase[28] (PHB depolmerase, 0.1 mg/mL) or trypsin[29] (10 mg/mL). After incubation for a requisite time (12 h or 24 h), the samples were washed with distilled water and then lyophilized for 24 h. Morphological changes were observed with a field-emission scanning electron microscope (FE-SEM, Hitachi S-4300).

Cell Adhesion. In order to examine the interaction of nanofiber mats with cells (NIH 3T3), the circular nanofibrous mats were fitted in a 24-well culture plate and subsequently immersed in a DMEM medium containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin G-streptomycin. To seed the cells, 300 μL of NIH 3T3 cell solution (5×10^4 cells/cm^2) was added and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After incubation for a 4 h, the medium solution was removed. These samples were washed twice with the PBS, and fixed by 2.5% glutaraldehyde aqueous solution for 20 min. The sample mats were then dehydrated in a graded concentration of ethanol (25, 50, 75, 90, and 100) for 10 min each. Finally, the sample mats were air dried in a fume hood overnight. Dry cellular structures were sputter-coated with gold and observed with FE-SEM.

BrdU Assay. The proliferation of NIH 3T3 cells was determined using a colorimetric immunoassay. This ELISA method was based on the measurement of 5-bromo-2-deoxyuridine (BrdU), which was incorporated during DNA synthesis.[30-32] The ELISA was performed according to the manufacturer's instructions (Roche Molecular Biochemicals).

Statistical Analysis. Results are displayed as mean ± standard deviation. Statistical differences were determined by a student's two-tailed *t*- test. Scheffe's method was used for multiple comparison tests at a level of 95%.

Results and Discussion

Biodegradable polymers have gained considerable importance in the biomedical industry as scaffolds and in the development of biomaterials for therapeutic applications. In particular, PHBV has received enormous attention regarding its biomedical application potential, which is, in part due to its ability to achieve desired mechanical properties and proper rate of degradation.[33]

Preparation of Modified Keratin. A distinctive feature of keratin is the occurrence of a large amount of cystein residues that are mainly present as the disulfide bonded dimeric amino acid cystine. Due to this extensive cross-linking and a high amount of hydrophobic residues, keratin is insoluble in polar solvents such as water, as well as in apolar solvents. Since the 1940s, several processes have been described in literature with regard to dissolve feather keratin. In our study, we achieved a clear keratin solution after the dialysis for two days. The removal of 2-mercaptoethanol and urea from this solution by dialysis resulted in the aggregation of keratin polypeptide chains and reoxidation of the cysteine residues that yielded a white, opaque gel.[34] Therefore, the sulphydryl groups in the solution were protected by iodoacetic acid before further work can be carried out. This is because such groups are extremely active and can be oxidized by atmospheric oxygen to form new disulfide bridges. According to our data, the modification degree of sulphydryl is nearly 100% when the molar ratio of IAA/SH is set at 5 (data are not shown).

Preparation of Electrospun Nanofibrous Mats. A series of ratios between PHBV and *m*-keratin (10/0, 7/3, 3/7, 0/10) were dissolved and electrospun. When electrospun *m*-keratin (10/0) using HFIP, the resulting fibers contained many beads. This is due to broad molecular weight distribution and low dissolvability of *m*-keratin (Figure 2(a)). Fibers that had good image were obtained when co-electrospinning *m*-keratin with PHBV. Figures 2(b), (c) and (d) showed the SEM images of the electrospun PHBV, PHBV/*m*-keratin (7/3), and PHBV/*m*-keratin (3/7) nanofibrous mats that were obtained under optimum conditions. The diameters of PHBV, PHBV/*m*-keratin (7/3) and PHBV/*m*-keratin (3/7) fiber were about 815±98, 720±124 and 487±161 nm, respectively. These data were calculated by Image J 1.38 software (http://rsb.info. nih.gov/ij/download.html). It is well known that polar polymer has a higher conductivity than non-polar polymer. Keratin is a polar biopolymer that contains many polar groups such as amide and carboxyl groups. When PHBV is blended with keratin and electrospun, the conductivity of solution will increase, thus leading smaller diameter of PHBV fibers. To maintain the mechanical property of PHBV/keratin mats, PHBV/*m*-keratin (7/3) was used for further test.

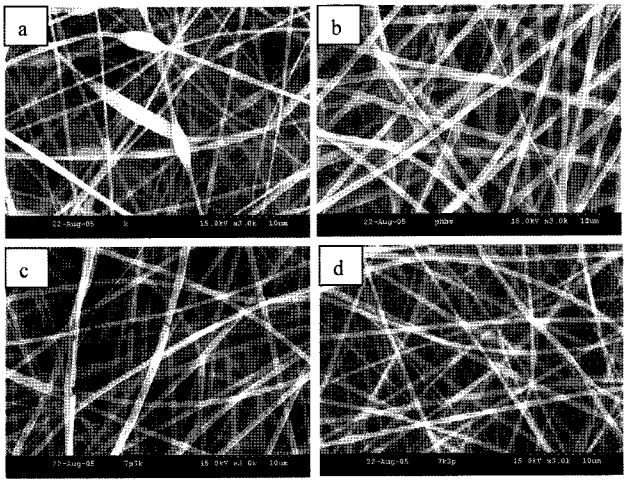

Figure 2. SEM images of nanofibrous mat: (a) 6% *m*-keratin in HFIP; (b) 6% PHBV in HFIP; (c) PHBV/*m*-keratin=7/3, 6% in HFIP; (d) PHBV/*m*-keratin=3/7, 6% in HFIP.

Crosslinking of *m*-Keratin Nanofibers. The as-spun keratin fibers can be easily dissolved in water. Therefore, *m*-keratin fibers need to be cross-linked to reduce their solubility. The most popular cross-linking reagent used in proteins is glutaraldehyde (GA). In order to block residual aldehyde group, glycine was used after treatment of *m*-keratin nanofibers with glutaraldehyde vapor. Figure 3 showed the effect of

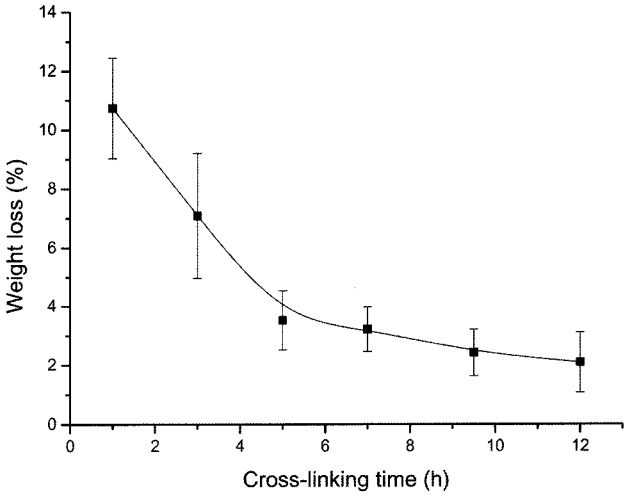

Figure 3. Weight loss of the *m*-keratin nanofibrous mats with cross-linking time.

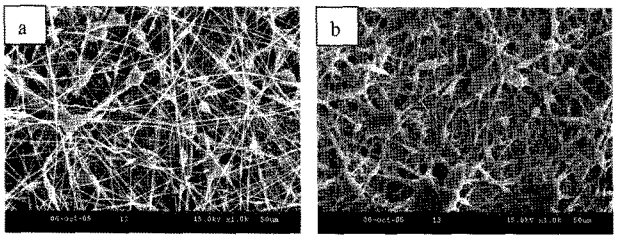

Figure 4. SEM images of *m*-keratin nanofibrous mats before (a) and after (b) crosslinking with glutaraldehyde vapor for 7 h.

cross-linking time on weight loss. After a reaction for 7 h, weight loss was almost constant. Therefore, 7 h was determined as an appropriate time. Figure 4 showed the morphology of the cross-linked keratin. Obviously, the *m*-keratin mat lost its fibrous form after surface crosslinking.

Characterization of Nanofibrous Mats. The water contact angle of PHBV film, PHBV nanofiber mats, as determined by the sessile drop method with contact anglemeter (Erma contact anglemeter, G-1 type, Japan), was 108° and 110°, respectively.[35] It was indicated that PHBV had a high hydrophobic nature. As expected, PHBV/*m*-keratin mats showed much better wettability than PHBV due to the introduction of *m*-keratin. The water drops on the PHBV/*m*-keratin disappeared after a few seconds. This indicated that the PHBV/*m*-keratin nanofibers had much better hydrophilicity than the PHBV mats. This property will be very helpful for cell adhesion. The ATR-FTIR spectra of the PHBV fibers (a), *m*-keratin fibers (b) and PHBV/*m*-keratin (c) are shown in Figure 5. The strong absorption peak at 1725 cm^{-1} is attributed to the ester groups of PHBV (Figure 5(a)). Figure 5(b) illustrates that common bands of *m*-keratin appeared at approximately 1650 cm^{-1} (amide I) and 1545 cm^{-1} (amide II), corresponding to the stretching vibration of C=O bond (amide I), the coupling of bending of N-H bond (amide II) and the

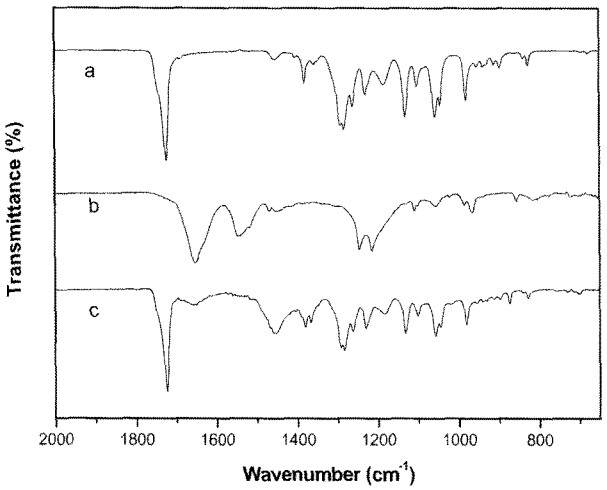

Figure 5. The ATR-IR spectra of (a) PHBV, (b) *m*-keratin, (c) PHBV/*m*-keratin=7:3.

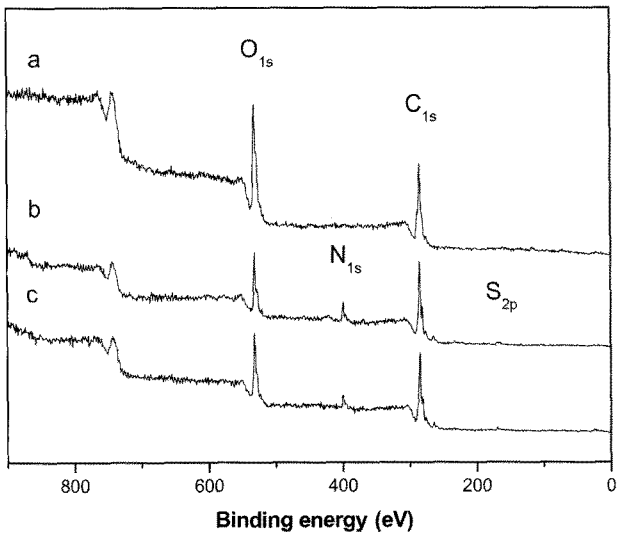

Figure 6. Electron spectroscopy for chemical analysis (ESCA) survey scan spectra of nanofiber mat (a: PHBV; b: *m*-keratin; c: PHBV/*m*-keratin=7/3).

stretching of C-N bond, respectively. The amide I bond absorption at 1650 cm^{-1}, that appeared in the PHBV/*m*-keratin spectrum (Figure 5(c)), was obviously due to the presence of keratin. When blended and electrospun with PHBV, the peak of amide I did not shift, indicating that the conformation of *m*-keratin was maintained.

Changes in the chemical structure of nanofibers were investigated with the ESCA (Figure 6). Compared with PHBV, the newly-appeared peaks at 400 eV in the PHBV/*m*-keratin obviously could be attributed to nitrogen of keratin. The sulfur peak in PHBV/*m*-keratin was very weak due to an extremely insufficient amount of content.

***In vitro* Biodegradation.** The morphology of the mats degraded *in vitro* was examined with the FE-SEM. Figure 7 illustrates the morphological changes of the electrospun mats during *in vitro* degradation. After 12 h of degradation in the PHB depolymerase aqueous sollution, a large part of the nanofibers disappeared; only chunks of degraded materials remained (Figures 7(c) and (d)). The fiber-binding points were broken, and the mat was found to be rather brittle and powdered. Fibrous morphology almost not changed when the mats of PHBV and PHBV/*m*-keratin were subjected to degradation in a trypsin solution for 12 h. Therefore, we extended the incubation time in a trypsin solution to 24 h. As the results, the degradation was also slightly observed in PHBV mat and some fibers began to break down (Figures 7(e) and (f)). It can be seen that the fibers had been severely eroded by the treatment of PHB depolymerase, while little erosion was found in the trypsin solution. These results indicated that PHB depolymerase was more sensitive to PHBV than to keratin. The enzymatic degradation of PHBV films by PHB depolymerase has been reported.[36] It is commonly accepted that *m*-keratin could be degraded in a trypsin solu-

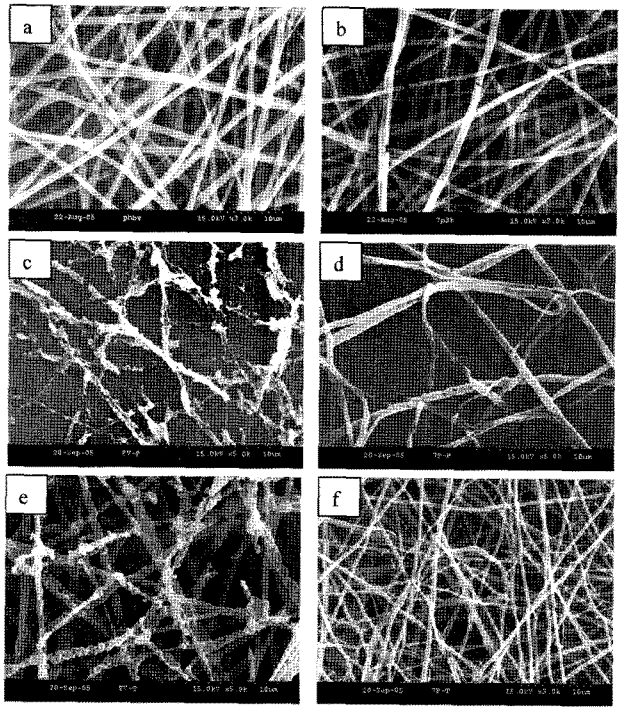

Figure 7. SEM images of the nanofibrous scaffolds incubated in the PHB depolymerase solution and the trypsin solution. (a) PHBV original, (b) PHBV/*m*-keratin=7/3 fiber original, (c) PHBV fibers incubated for 12 h after PHB depolymerase aqueous solution, (d) PHBV/*m*-keratin=7/3 fibers incubated for 12 h after the PHB depolymerase aqueous solution, (e) PHBV fibers incubated for 24 h in trypsin aqueous solution, (f) PHBV/*m*-keratin=7/3 fibers incubated for 24 h in trypsin aqueous solution.

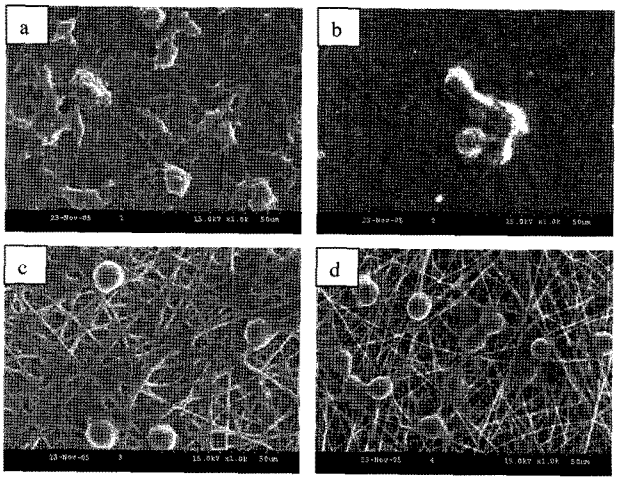

Figure 8. SEM micrographs of NIH 3T3 cells cultured for 4 h on electrospun nanofibrous scaffolds: (a) *m*-keratin fibers, (b) PHBV film, (c) PHBV fibers, and (d) PHBV/*m*-keratin=7/3 fibers.

tion.[9]

Cell-Scaffold Interaction. Ideal tissue engineering scaffold material must support cellular attachment and growth. To evaluate cellular behavior on electrospun fibers, fibroblasts were seeded and cultivated. Figure 8 shows the SEM images of cells that adhered to the nanofibrous mats when cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum for 4 h. As expected, on the surface of all electrospun fibers, more cells were attached and showed much more spread morphology than that of the PHBV film at an earlier culture stage (4 h). In spite of hydrophobic nature of PHBV, cells more adhered onto PHBV fibers than PHBV film, due to their three-dimensional structures. These fibroblasts interacted and integrated well with the surrounding fibers. The cells that were barely adhered to the PHBV film had more of a round shape. SEM micrographs showed that the development of cells growth was guided by fibrous architecture. Cells grew in the direction of the fiber orientation, and then formed a three-dimensional and multicellular network, according to the architecture of the nanofibrous structure. During the cells stayed on the surface of the fibers, some cells migrated underneath. However, after 1 d culture, the cells grew among the fibers and covered the whole surface (data are not shown).

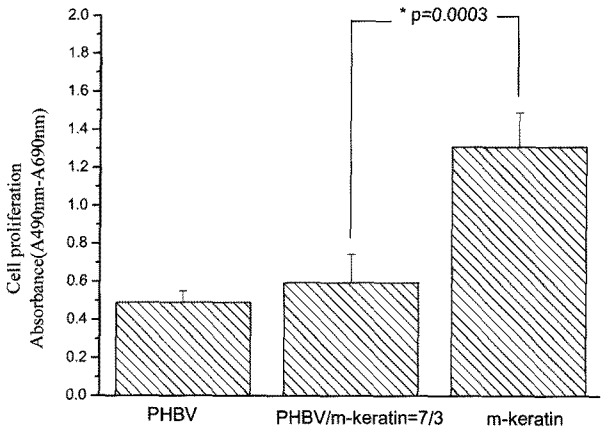

Figure 9. The proliferation of NIH 3T3 cells cultured for 20 h. (Data are expressed as means ± SD (n=6) for the specific absorbance, * $p<0.01$, values are significantly different from those of the previous group).

Figure 9 shows the proliferation of cells on the nanofibrous mats when cultured in DMEM medium with 10% serum for 20 h. *m*-Keratin fibers exhibited the highest cell proliferation potential when compared to the PHBV or PHBV/*m*-keratin fibers. Cell proliferation on *m*-keratin fibers was significantly different from that of PHBV fibers and PHBV/*m*-keratin fibers. However, there is no significantly difference between PHBV/*m*-keratin and PHBV control due to low content of *m*-keratin. It is reasonably concluded that proliferation of NIH3T3 cells was accelerated by *m*-keratin proteins.

Conclusions

Keratin is one of the most important proteins for wound

healing and tissue recovery. In this study, the modified keratin was prepared and coelectrospun with PHBV to obtain the PHBV/*m*-keratin composite nanofibrous mats. These biodegradable keratin nanofiber mats could accelerate the adhesion and proliferation of NIH 3T3 cells as compared to PHBV control. PHBV/*m*-keratin composite nanofiber mats would be good candidates for biomedical applications, such as wound dressing and scaffolds for tissue engineering.

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