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The polymeric crystallinity effect on the responses of bone marrow stromal cells

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Abstract: The cell-material interaction is an important factor to be considered in the applications of biomaterials. The properties of biomaterials, for example crystallinity, would change the cell behaviors such as proliferation, differentiation, morphology and so on. In this article, the research was focused on the relationship between the crystallinity of polymeric substrates and the cell responses. Two kinds of biodegradable poly(L-lactide) (PLLA) films with different crystallinity were prepared and used as substrates for cell behavior study. The PLLA films were fabricated with crystallinity of 19% and 69% through solution casting and isothermal processing, respectively. The crystallinity of PLLA films was characterized by DSC and XRD. The surface morphologies of PLLA films with different crystallinity were observed by SEM. Bone marrow stromal cells (MSCs) isolated from the marrow of new born male Sprague Dawley rats were cultured on the films. The cell morphology, proliferation, total protein amount and alkaline phosphatase (ALP) activities were tested. The results showed that the crystallinity of PLLA films affected the behavior of MSCs greatly.

Keywords: PLLA, crystallinity, bone marrow stromal cells, cell-biomaterials interaction

Introduction

Applications of biodegradable polymeric materials in tissue engineering, regenerative medicine and implants, have recently raised increasing interests for biomaterials scientists [1-3]. Cell-biomaterials interaction was a general and unavoidable process when cells came in contact with biomaterials. The properties of biomaterials such as chemical compositions, morphologies, phase components and crystallinity played an important role in the cell-biomaterials interactions. For example, nonporous or porous substrates affected the cells expression of phenotypes [4]. The porosity and pore morphology of tissue engineering scaffolds decided the attachment of cells [5]. The modifications of polymeric substrates would change the initial steps of cells deposition and affinity [6-9]. Thus, the detailed study of biomaterials' properties effects on cell responses is worth exploring.

Poly(L-lactide) (PLLA) is a primarily used biomaterial due to its excellent physical properties, biocompatibility, biodegradability and low immunogenicity both *in vivo* and *in vitro* [10-15]. The remarkable results were that PLLA made contributions to promote the differentiation of osteoblast-like cells and enhance bone formation [7, 16-18]. As a semi-crystalline polymer, the mechanical, processing and degradation properties of PLLA were largely dependant on its crystallinity. It was reported that the *proteinase K* preferred to hydrolyze the amorphous region rather than the crystalline region of

PLLA [19, 20]. The mechanism of the crystallinity effect on cell responses and crystallinity effect on cells is unknown. The discovery of the relationship between crystallinity and cell responses will contribute to the study of cell-biomaterials interaction both in theoretical foundations and technical progresses. Though many papers carried out the process to characterize the crystallinity of PLLA, there were rare reports on the cell responses to the polymeric crystallinity.

In this paper, the research was focused on the crystallinity effect on cell-biomaterials interactions. PLLA was fabricated into films with different crystallinity. The properties of PLLA films were tested by automatic polarimeter, DSC, XRD and SEM. Bone marrow stromal cells (MSCs) were cultured on PLLA films and their behaviors such as adhesion, proliferation and early differentiation were studied *in vitro*. In addition to cell morphologies and proliferation examined by SEM and MTT assay, total protein amount and alkaline phosphatase (ALP) activity as the growth and differentiation functions were measured.

Results and discussion

Properties of PLLA

The optical purity of PLLA was determined by an automatic polarimeter at a wavelength of 589 nm. The a_D^{25} value was approximately -159°, which was according to that in reference [21].

Tab. 1. Thermal properties of h-PLLA and l-PLLA films.

Samples	T_g(℃)	T_m(℃)	ΔX_c %
h-PLLA	69.72±1.06	178.81±0.86	69.30±0.51
l-PLLA	57.76±1.05	176.13±0.92	19.01±0.84

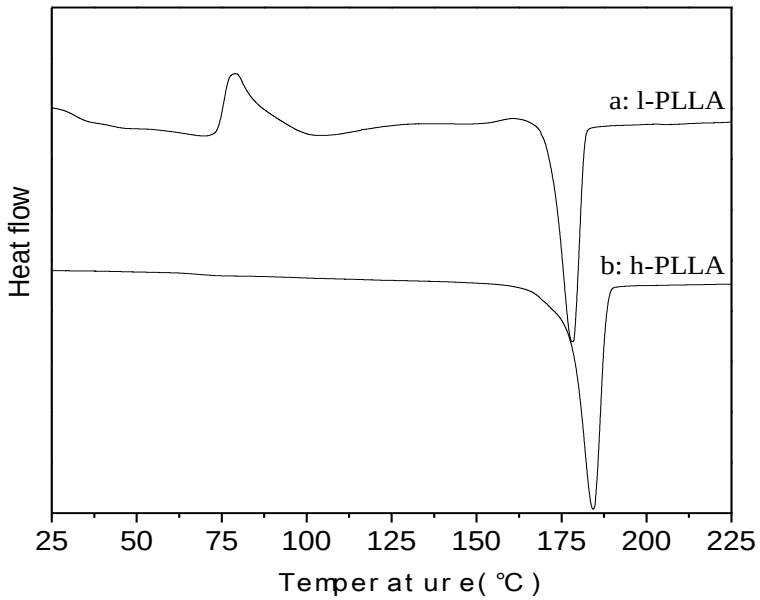


Fig. 1. DSC thermogram of l-PLLA and h-PLLA films.

Thermal properties of PLLA films with high (h-PLLA) and low crystallinity (l-PLLA) were determined by DSC in nitrogen atmosphere. The glass transition temperature (T_g), melting point (T_m) and degree of crystallinity (X_c) are illustrated in Tab. 1. The X_c was calculated by $X_c = (\triangle H_m - \triangle H_c) / \triangle H_{m,100\%}$, where the $\triangle H_{m,100\%}$ was 93.7 J/g [22]. According to the formula, the high crystallinity of PLLA was 69%, which was much higher than that of l-PLLA.

The DSC spectra of PLLA films are presented in Figure 1. Contrast to the spectrum of h-PLLA, the spectrum of l-PLLA exhibited a lower T_m. An exothermic crystallization peak appeared which represented the cold crystalline process during the heat scan.

X-ray diffraction also demonstrated the differences between the two types of PLLAs (Fig. 2). In the h-PLLA spectrum, the strong diffraction peaks primarily appeared at 2θ around 16.60° and 19.04°. While the diffraction peaks of l-PLLA were much weaker and the amorphous area was obvious. Both h-PLLA and l-PLLA showed α-crystalline structure [23, 24].

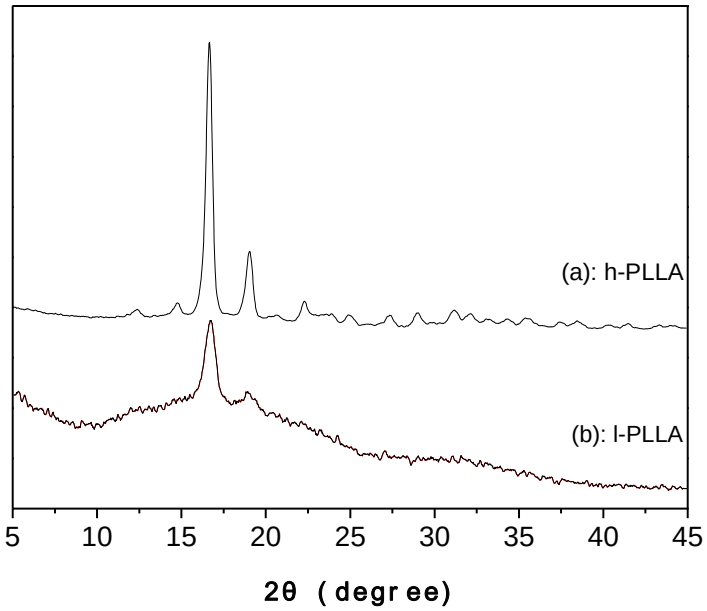
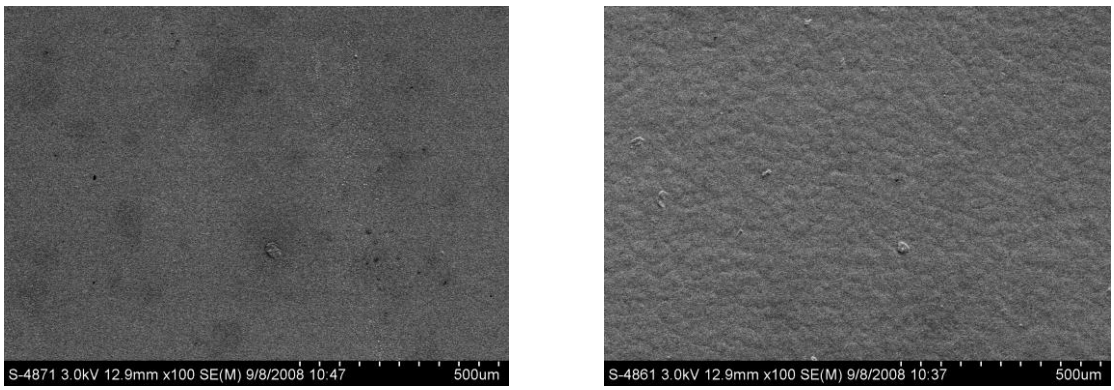


Fig. 2. XRD profiles of (a) h-PLLA and (b) l-PLLA films.



(a) (b)

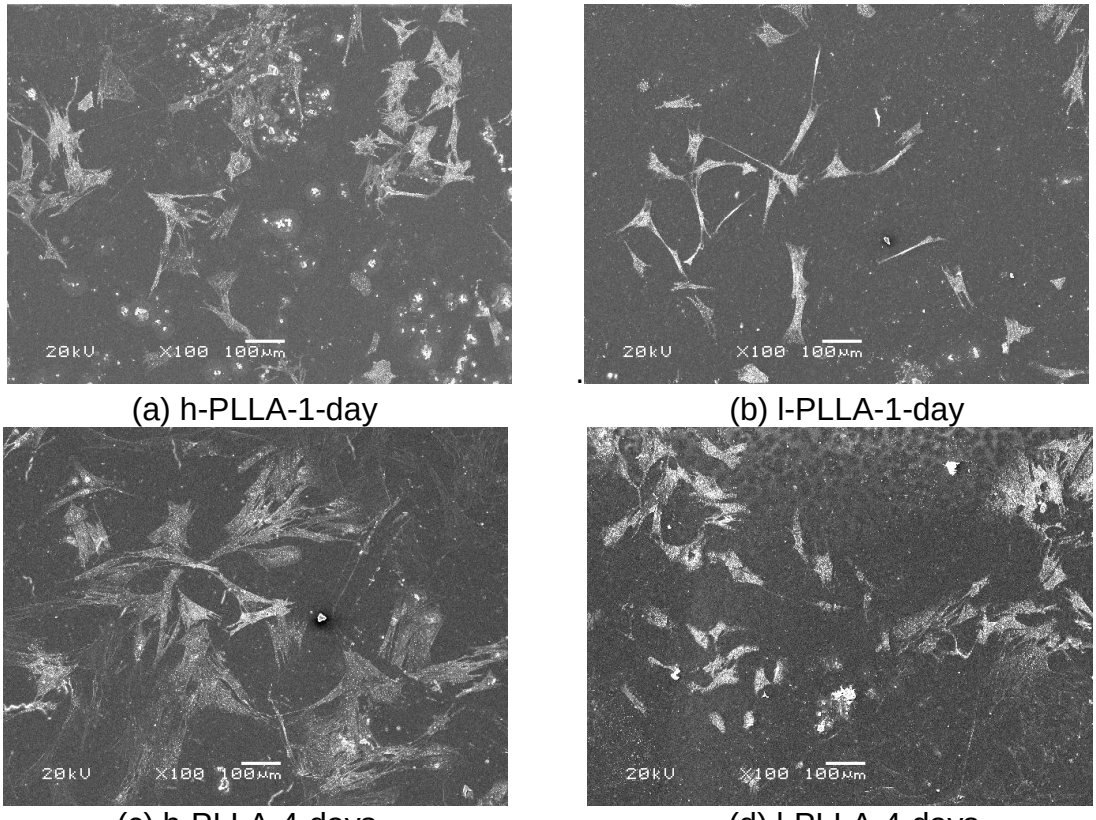
Fig. 3. SEM images of (a) h-PLLA and (b) l-PLLA films

Fig. 3 showed the surface morphologies of l-PLLA and h-PLLA films. It was clear that the h-PLLA surface was smoother than that of l-PLLA. It was attributed to the high crystal density by the treatment of isothermal crystallization [25]. The isothermal treatment reorganized the PLLA chains and increased its bulk crystallinity, sequentially promoting the smoothness of surface [26].

Evaluation of MSCs on PLLA films

In order to study the cell responses on PLLA films, MSCs were seeded on PLLA films to investigate the cell proliferation, phenotypes (ALP activity) and cellular phenomenological behavior.

MSCs on PLLA films were immobilized and observed. The MSCs cultured on the h-PLLA and l-PLLA films after 1 and 4 days incubation are shown in Fig. 4. With 1 day cultivation, the cells on h-PLLA films showed fibroblast-like monolayer morphologies, but those on l-PLLA seemed slimmer. After 4 days cultivation, the cells on h-PLLA films exhibited flattened and overlapped multilayers. Though the MSCs on l-PLLA also showed expanding morphology, the cells were smaller than those on h-PLLA. From the photographs, it could be concluded that the MSCs were much preferred to attach and proliferate on h-PLLA film. These results implied that the cell morphologies were influenced by the crystallinity of PLLA films to a certain extent.



(a) h-PLLA-1-day (b) l-PLLA-1-day

(c) h-PLLA-4-days (d) l-PLLA-4-days

Fig. 4. SEM photographs of MSCs cultured on h-PLLA and l-PLLA films after 1 and 4 days incubation, respectively.

The effects of crystallinity on the proliferation of MSCs were investigated by MTT assay. Fig. 5 shows the growth of MSCs on each film after 1-14 days incubation.

Within all the 14 days, the number of MSCs grown on each film increased with incubation time increasing. The cell proliferation showed nearly no difference between h-PLLA and l-PLLA within the first 2 days. From the beginning of 4 days, the cells on h-PLLA proliferated much faster than those on l-PLLA, and the statistical differences was perceptible (p=0.043). The differences appeared obvious with incubation time increasing (7 days, p=0.006). After incubated for 14 days, the statistical difference lowered (p=0.039). It was because the cells overspread the culture plates and thus led to the statistical difference lowering down. From the results of cell proliferation, it was clear that the crystallinity affected the behavior of MSCs and the viability of MSCs on h-PLLA was higher than that on l-PLLA.

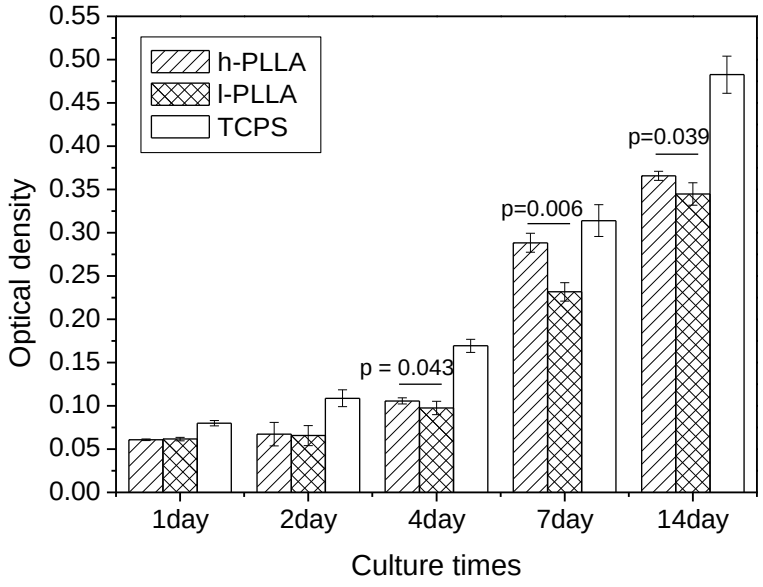


Fig. 5. The proliferation of MSCs on h-PLLA and l-PLLA films, determined by MTT assay (Error bars designate means ±SD for n = 3).

The total protein amount was directly associated with the number of cells, which reflected the growth of MSCs. As shown in Fig. 6, the total protein amount on both films increased gradually during the culture time. The variation trend was the same as cell proliferation trend. The statistic difference of total protein amount between h-PLLA and l-PLLA also appeared at the fourth day. The total protein amount on h-PLLA film was higher than that on l-PLLA. This demonstrated that the cells preferred to grow on h-PLLA. This result was consistent with that of MTT assay.

Alkaline phosphatase of MSCs, which was considered as a marker of an early stage of osteocyte differentiation, was measured on both film surfaces (Fig. 7). The ALP activity expression increased with culture time increasing, just like Ishaug reported [18]. The ALP activity measured on all substrates almost maintained at the same level within the first 4 days; it increased slightly after 7 days. However, ALP activity increased significantly after culturing for 14 days. The ALP activity on h-PLLA showed statistical difference and was higher compared to that on l-PLLA after 7 days (p=0.041). After 14days, ALP activity on h-PLLA showed prominent growth (p=0.001).

Alkaline phosphatase reflected an inverse relationship between cell proliferation and differentiation [20]. Based on the results of Fig. 5 and Fig. 7, the proliferating MSCs

demonstrated low expression of their typical phenotypic activities during the periods of rapid growth, and while the cell replication slowed down, the production of ALP increased. One can see clearly that the optical density values increased 160% and 125% for h-PLLA and l-PLLA, and the ALP activity increased 100% for h-PLLA and 80% for l-PLLA, respectively. At the later stage of incubation (7-14 days), optical density values increased 25% and 55% for h-PLLA and l-PLLA respectively, but the ALP activity increased remarkably 325% for h-PLLA and 150% for l-PLLA.

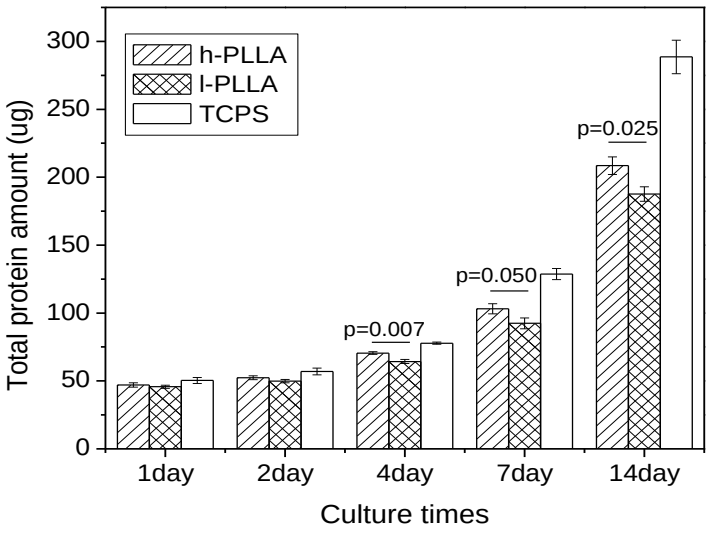


Fig. 6. Total protein amount of MSCs cultured on h-PLLA and l-PLLA films after 1, 2, 4, 7 and 14 days in culture (Error bars designate means ±SD for n = 3).

According to these results, it was found that the cell responses were dependant on the crystallinity of polymeric substrates. Park and Cima [26] have found that the hepatocytes response was sensitive to the poly(L-lactide) with different bulk crystallinity (13% and 37%).

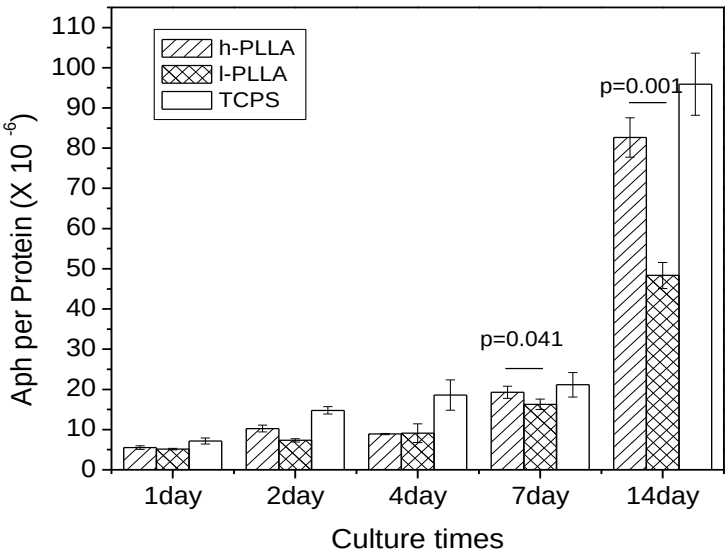


Fig. 7. ALP activity expressed by MSCs cultured on h-PLLA and l-PLLA films after 1, 2, 4, 7 and 14 days in culture (Error bars designate means ±SD for n = 3).

They investigated series of potential causes, such as changes in surface structure that occurred in the annealing process, release rate of crystalline, amorphous substrates degradation products, substrate surface free energy and ageing of amorphous regions, but no powerful evidence or precise measurable method could be concluded. In this paper, we studied the effect of crystallinity on cell responses and found the proliferation and differentiations of MSCs were affected by the crystallinity.

Conclusions

PLLA films with different crystallinity (19% and 69%) were prepared. The effects of crystallinity on the physicochemical and surface properties of polymeric films as well as MSCs attachment, proliferation and differentiation behaviors were investigated. The PLLA with high crystallinity showed better properties in the morphology, proliferation, total protein amount and ALP activity expression of MSCs. Thus, the results implied that the crystallinity of polymeric substrates affected the cell-material interactions remarkably. Further research on the relationship between crystallinity and osteogenesis differentiation is under investigation.

Experimental part

Preparation of polylactide films

L-lactide (Shenzhen Yisheng new materials Co., Ltd, China.) was recrystallized twice in ethyl acetate and toluene, respectively. PLLA was synthesized by ring-opening polymerization in the presence of stannous octoate (Sigma, USA) as a catalyst. The viscosity average molecular weight of PLLA was 100KDa.

PLLA films were prepared by casting 2.5wt% of poly(L-lactide)/chloroform solution on glass petri dishes. All of the obtained films were then dried in air for 3 days, and further vacuum-dried for 3 days.

The solvent-cast was used to prepare l-PLLA film. PLLA with high crystallinity was processed by isothermal crystallization at 140 ^{0}C for 2 hour in a vacuum oven (0.001Torr) [26]. The films were cut into pieces with 14 mm diameter before use, and the surface to glass was used for the subsequent experiments.

Characterization of the polymer

The specific optical rotation a of the polymers was measured in chloroform at a concentration of 1g/dL at 25 ^{0}C using an automatic polarimeter (AUTOPOL V) at a wavelength of 589 nm. The differential scanning calorimetric (DSC) measurements were performed on a TA system Q2000 under nitrogen at a flow rate of 50mL/min. Each sample was heated from 25 to 225 ^{0}C at a heating rate of 10 ^{0}C /min. X-ray diffractometry (XRD) measurements were carried out at room temperature on an X' Pert Pro X-ray diffractometer with a Cu Kα (v=1.54056nm) radiation source. Morphologic evaluation of the film surfaces was carried out with SEM (Hitachi, S4800).

Cell harvesting and culture

Bone marrow stromal cells (MSCs) were obtained from the marrow of new born male Sprague Dawley rats. Briefly, following death by decapitation, femora and tibiae were

aseptically excised and the soft tissue was cut off. After cutting off the bone ends, the marrow was flushed out with a-Modification of Eagle's Medium (a-MEM) containing 20% fetal bovine serum (FBS, Beijing Biosynthesis Biotechnology Co., LTD, China) and antibiotics using a syringe equipped with a 16-gauge needle and collected in a sterile petri dish (FALCON, USA). The cell suspension was divided into several petri dishes and cultured at 37 ^{0}C in a 5% CO_2 atmosphere. After 3 days, impurity and other unattached cells were removed from the dishes by gently washing with PBS. The cells were cultured in a-MEM culture medium containing 15% FBS, antibiotics (100mg/mL penicillin and 100mU/mL streptomycin) and fresh prepared ascorbic acid (50 ug/mL, Sigma). After 2 weeks incubation, the cell monolayer (passage 3) reached about 90% confluence, and was used for further experiments.

Evaluation of MSCs on PLLA films

The polymeric films with 14 mm in diameter were sterilized under ultraviolet light and placed in 24-well tissue culture polystyrene plates (FALCON , USA). A glass cylinder was placed on each of the films to prevent them from floating. Subsequently, 1 mL medium of cell suspension at a concentration of 1.0×10^4 cells/mL was added to each well and maintained in a humidified atmosphere with 5% CO_2 at 37 ^{0}C. The cell suspension was also placed in empty tissue culture polystyrene dish (TCPS) with a glass cylinder as control. The culture medium was changed every 2 or 3 days.

Cell morphology

Cells adhered to the films were gently washed with PBS after 1 and 4 days incubation. The cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS for 2 h at 4 ^{0}C, rinsed twice with PBS and once with distilled-deionized water, stored in a deep freezer (–80 ^{0}C) after adding distilled-deionized water. Before SEM (Hitachi, S4800) observation, the fixed samples were freeze-dried and sputter coated with gold.

Cell viability

After the cells were cultured for 1, 2, 4, 7 and 14 days, the viability of MSCs was determined by MTT assay. MTT (Sigma) was prepared as a 5 mg/mL stock solution in PBS, sterilized by Millipore filtration, and kept in dark. The 100ul of MTT solution was added to each well. After 4 h incubation at 37 ^{0}C, 1 mL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The dissolvable solution was mixed homogeneously about 30 s by the shaker. The optical density (OD) of the formazan solution was read on a microplate reader (BIO-RAD, model 550) at 570 nm.

Total protein amount and alkaline phosphatase activity

On days 1, 2, 4, 7 and 14, after removal of the culture medium, the cells were rinsed twice with PBS, 500 ul distilled-deionized water was added, and the cells went through 5 cycles of freezing and thawing and centrifuged at 6700 G for 3 minutes. These supernatants were used as sample solutions for the measurements of total protein amount and ALP activity.

Total protein amount of cell lysates was measured according to Smith [27] , with an available BCA protein assay kit (Novagen, #71285-3). The absorbance at 570 nm was measured in micro- plate reader (BIO-RAD, model 550).

ALP activity was measured spectroscopically [28] using a commercial assay kit (ANASPEC, #71230). The ALP activity of cell lysate was normalized for total protein amount of the cell lysate.

Statistics

Statistical analyses were performed using Origins 6.1. One way analysis of variance (ANOVA) was used to determine statistical significance of differences in the parameters of cell growth. Similar results were obtained from at least two independent experiments. The results were expressed as mean ± SD of three parallel samples at least.

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