Enhanced human bone marrow stromal cell affinity on modified poly($_L$-lactide) surfaces by the upregulation of adhesion molecular genes

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ABSTRACT

To enhance and regulate cell affinity on poly ($_L$-lactic acid) (PLLA) based materials, two hydrophilic ligands, poly (ethylene glycol) (PEG) and poly ($_L$-lysine) (PLL), were used to develop triblock copolymers: methoxy-terminated poly (ethylene glycol)-*block*-poly ($_L$-lactide) –*block*-poly ($_L$-lysine) (MPEG-*b*-PLLA-*b*-PLL) in order to regulate protein absorption and cell adhesion. Bone marrow stromal cells (BMSCs) were cultured on different composition of MPEG-*b*-PLLA-*b*-PLL copolymer films to determine the effect of modified polymer surfaces on BMSC attachment. To understand the molecular mechanism governing the initial cell adhesion on difference polymer surfaces, the mRNA expression of 84 human extracellular matrix (ECM) and adhesion molecules were analyzed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). It was found that down regulation of adhesion molecules was responsible for the impaired BMSC attachment on PLLA surface. MPEG-*b*-PLLA-*b*-PLL copolymer films improved significantly the cell adhesion and cytoskeleton expression by upregulation of relevant molecule genes significantly. Six adhesion genes (CDH1, ITGL, NCAM1, SGCE, CoL16A1, and LAMA3) were most significantly influenced by the modified PLLA surfaces. In summary, polymer surfaces altered adhesion molecule gene expression of BMSCs, which consequently regulated cell initial attachment on modified PLLA surfaces.

INTRODUCTION

Tissue engineering holds great promise for the numerous patients who suffer tissue loss or organ failure, by delivering therapeutic cells via biomaterials to defect areas in order to replace lost tissue function. The attachment of mammalian cells onto natural or synthetic matrices is a crucial step for the implantation of cells and cell/matrix complexes. Mesenchymal stem cells are a population of non-haematopoietic cells that can be isolated from bone marrow and are capable of differentiating into osteoblasts, chondrocytes, and adipocytes when reintroduced *in vivo*. In order to create effective cell-based therapies, therapeutic cells need to be carried by biomaterials and a critical challenge to this approach is developing suitable matrix materials, since cell function and viability are greatly affected by the substrate. However, given the hydrophobic nature of most synthetic biodegradable polymers, anchorage-dependent cells such as bone mesenchymal stem cells do not attach and grow well on these materials compared with natural polymers such as type I collagen matrix.

For example, Poly ($_{L}$-lactic acid) (PLLA) is a versatile biodegradable polymer which is considered a promising material for tissue engineering applications. However, due to a lack of functional groups, PLLA cannot be modified easily with biologically-active moieties, whereas the hydrophobic surface of PLLA materials leads to poor cell adhesion and spreading [1, 2]. Many researchers have focused on modifying PLLA surfaces to achieve more cell-friendly properties, either through the design of functional block copolymers or binding of active molecules. Poly(ethylene

glycol) (PEG), a hydrophilic, water soluble and non-toxic polymer, has been used to modify PLLA in order to prevent protein non specific binding and achieve a hydrophilic surface to improve cell proliferation; however, the PLLA-PEG copolymer films did not enhance cell attachment efficiencies and, in fact, appears to decrease the extent of cell attachment [3]. Poly($_L$-Lysine) (PLL), a synthetic poly (α-amino acid) molecule, has also been applied with PLLA to enhance cell attachment, since the inclusion of functional side groups of amino acids into copolymers can improve the affinity of the materials for proteins or cells [4].

Adhesion to biomaterials is the primary step that influences cell behaviour and induces the cascade of events leading to new tissue formation [5, 6]. *In vivo* cell adhesion process is mediated by the interactions between cell adhesion molecules including integrins ,cadherins, and selectins, as we as adhesion proteins existing in the extracellular matrix (ECM) such as fibronectin, vitronectin, laminin and collagen [7]. *In vitro* cell adhesion and functions are thought to be influenced by the extracellular environment, especially the culture media and the properties of the material surface [8]. The surface of the material on which cells grow plays an important role in controlling cell behaviour in the initial period of cell-material contact [9, 10]. Hence, any modification of the material surface that improves the initial stage of cell adhesion is an important aspect in the design of new polymeric biomaterials for tissue engineering [10, 11]. However, the molecular mechanisms governing the initial cell and polymer surface interaction are not clear. In this study the initial cell adhesion and regulation of 84 cell adhesion molecule and ECM genes involved in the early cell

attachment were analysed on PLLA and PEG/PLL modified PLLA surfaces to unveil the molecular mechanism on cell adhesion, especially regulation of adhesion molecule genes on the amphiphilic triblock copolymers of methoxy-poly(ethylene glycol) -*block*-poly($_L$-lactide)-*block*-poly($_L$-lysine) (MPEG-*b*-PLLA-*b*-PLL) modified PLLA surfaces.

MATERIALS AND METHODS

Preparation of polymer films from PLLA and PLLA modified by MPEG$_{17}$-*b*-PLLA$_x$, PLLA$_x$-*b*-PLL$_y$, or MPEG$_{17}$-*b*-PLLA$_x$-*b*-PLL$_y$ copolymer

Diblock (MPEG$_{17}$-*b*-PLLA$_x$, PLLA$_x$-PLL$_y$) and triblock (MPEG$_{17}$-*b*-PLLA$_x$-*b*-PLL$_y$) copolymers were synthesized according to the procedures reported in our previous study [12]. Triblock polymer films were prepared by dissolving commercial high molecular weight PLLA (M_w=100,000-150,000g/mol) and the triblock copolymers of MPEG-*b*-PLLA-*b*-PLL in chloroform (5 wt. % of MPEG-*b*-PLLA-*b*-PLL being blended with 95% PLLA), casting the solution onto glass dishes (35 mm in diameter) and allowing the solvent to evaporate completely in air. Films containing the diblock copolymers MPEG-*b*-PLLA or PLLA-*b*-PLL and pure PLLA films were prepared the same way and used as controls. Tissue culture polystyrene (TCPS) were severed as positive control. The polymer films were sterilized by UV light for 20 minutes, cut into round pieces 5 mm across and placed into the wells of 96-well tissue

culture plates, and then washed three times with sterile phosphate buffered solution (PBS).

Cell culture

The use of human BMSCs was granted by the Queensland University of Technology ethics committee. The cells were isolated from human bone marrow samples as described previously [13, 14]. Briefly, samples were placed in 50 mL Falcon tubes containing 5 ml PBS supplemented with 200 U /mL heparin. After filtering through a 100 µm filter, the samples were mixed with Hanks buffer (GIBCO, Invitrogen Corporation, Melbourne, Australia) and then 15 mL of lymphoprep (Aix-Shield PoC AS, Oslo, Norway) was gently layered on top (15 mL Lymphoprep per 30 mL sample). The samples were then centrifuged at 400 x *g* for 35 minutes at 20 ºC without acceleration or braking. Cells located at the interface between the bone marrow sample and lymphoprep were collected and further re-suspended in 1 mL of Dulbecco's Modified Eagle's Medium with low glucose (DMEM-LG; GIBCO) supplemented with batch tested 10% (v/v) foetal calf serum (FCS) (HyClone, Logan, UT), 10 U/mL penicillin G, and 10 µg/mL streptomycin (GIBCO). Cells were cultured in a humidified incubator at 37 ºC containing 5% CO_2/95% atmospheric air. Hematopoietic cells were removed by media changes and adhesion BMSCs at passage three to six were used in this study.

Cell attachment assay

The BMSCs were collected by trypsinizing, centrifuging, and washing once with serum-free DMEM. 100 μL of DMEM was added to each well to keep the polymer film on the bottom of the well. Approximately 7,000 cells were seeded in each well in either 10% FCS or FCS-free medium. After 1 hour incubation at 37 ºC, unattached cells were removed by rinsing three times with PBS. The total number of cells adhered to the films was quantified using the CyQuant NF Cell Proliferation Assay Kit (Invitrogen, Australia) following the manufacturer's instructions, and samples were read using a fluorescence microplate reader (Polarstar Optima, Germany) with the excitation wavelength at 485 nm and emission detection at 585 nm [11, 15].

Cell morphology in initial adhesion

Cell morphology studies during the early adhesion process were performed by serum starving BMSCs for 4 hours and then seeding the BMSCs on the polymer films in serum supplemented culture media. After 1 and 2 hours incubation the samples were washed three times with PBS then fixed in 4% glutaraldehyde buffered in 0.1 M sodium cacodylate. Then the cells were post-fixed in 1% osmium tetroxide for 1 hour and then dehydrated through a series of graded ethanol solutions and finally critical point dried in hexamethyldisilazane (HMDS). The films were gold sputtered in a vacuum and then viewed with a SEM (FEI Company, USA) at an accelerating voltage of 10 kV [16].

Cytoskeleton organization

Actin fibre distribution in the cells on the polymer films was observed by serum starving the cells for 4 hours prior to seeding on the polymer films. The cells were incubated for 1 and 24 hours, the attached cells then rinsed with PBS, fixed with 4% paraformaldehyde for 20 minutes and then rinsed with PBS. The cells were permeabilized in 0.2% Tween20 in PBS for 20 minutes, and then incubated with 0.1mg/mL propidium iodide (Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 minutes to stain the cell nuclei. After being washed three times with PBS, the cells were incubated in phalloidin Alexa Fluor 488 (Invitrogen, Australia) for 30 minutes to stain filamentous actin, washed three times with PBS and mounted in 10 μL of mounting solution (Prolong Gold, Invitrogen). Images were captured with a confocal laser scanning microscope (Leica TCS SP5, Germany).

Gene expression by qRT-PCR analysis

The expression of 84 ECM and adhesion molecule genes in BMSCs attached on polymer surfaces was analysed using RT^2 Profiler™ PCR Array for extracellular matrix & adhesion molecules (SuperArray Bioscience Corporation, Frederick, MD, USA) following the manufacturer's protocol. Briefly, BMSCs were seeded on $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107}, PLLA and TCPS surfaces. After 24hrs incubation, total RNA was isolated from cells using TRIzol reagent (Invitrogen, Australia). The RNA sample was evaluated spectrophotometrically at 260/280nm and all samples had

absorbance ratios (260/280nm) between 1.8 and 2.0 with prominent 18s and 28s bands. Total RNA was used as template for first strand cDNA synthesis which was then diluted and added to a master mix containing fluorescent SYBR green dye. Aliquots were distributed across a 96-well plate, each well containing pre-dispensed extracellular matrix & adhesion molecule gene primer sets, and real-time PCR performed on a thermal cycler (7300 ABI Prism, USA). The conditions for PCR were as follows: 95^{o}C for 10min for activation, followed by 40 cycles of denaturation step of 95^{o}C for 15sec and primer extension at 60^{o}C for1 min. The plates contain a panel of housekeeping gene to normalize the PCR array data, as well as estimate the linear dynamic range of the assay. All experiments were repeated at least three times for each sample. Relative gene expression was calculated using the ΔΔCt method to compare mRNA expressions between TCPs, PLLA and $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107} modified PLLA surfaces. Gene expressions increased or decreased with a p value less than 0.05 was considered significantly differential expression. All the data was analysed using the Superarray software available at the website www. Superarray.com. The ontology of the adhesion genes were classified into functional families to review their biological function.

Statistical analysis

Statistical evaluation of the data was carried out by one-way analysis of variance (ANOVA) and by Scheffe's test. A level of significance was set at $P<0.05$. Original data were collected and normalized according to the cell numbers, cell percentages

and fluorescence values. Statistical analysis was performed to compare the variations within multiple groups.

RESULTS

The early attachment of BMSCs to the polymer surfaces was evaluated by measuring the relative number of attached cells on each membrane after one hour incubation by quantifying the amount of fluorescent dye binding to total DNA. The ratio of the relative number of attached cells on the modified PLLA membranes versus PLLA membranes in the presence or absence of serum was calculated (Figure 1-3).

Triblock copolymer significantly enhanced initial cell attachment

In the diblock copolymer films, the effect of MPEG on cell attachment was significantly influenced by the presence of serum. In the culture containing 10% FCS the content of MPEG in the diblock copolymer appeared to have no effect on cell attachment compared with PLLA alone. In serum-free culture conditions, with increasing MPEG content in the diblock copolymers, greater cell attachment was observed (Figure 1). The number of cells attached on the diblock copolymer films $MPEG_{17}$-*b*-$PLLA_{94}$ and $MPEG_{17}$-*b*-$PLLA_{56}$ was significantly higher than pure PLLA film. TCPS provided a positive control in FCS showing increased cell adhesion and a negative control in serum-free conditions with decreased cell attachment.

The effect of PLL on cell attachment was observed in serum and serum-free conditions. The result showed that cell attachment was unaffected by the presence of serum or not. Both $PLLA_{139}$-*b*-PLL_{131} and $PLLA_{77}$-*b*-PLL_{72} copolymer films showed an increased cell attachment in serum and serum-free conditions compared with PLLA film. (Figure 2).

The triblock copolymer $MPEG_{17}$-*b*-$PLLA_x$-*b*-PLL_y was synthesised in order to study the effect of the combination of MPEG and PLL on PLLA surfaces. The results showed that regulation of cell attachment depended on the right combination of the three polymer components. In general significant upregulation of cell attachment was observed in the triblock copolymer compared with PLLA alone or MPEG-*b*-PLLA diblock copolymer, in both serum and serum-free conditions. There was a clear trend of increased cell attachment with increase in PLL content in the triblock polymers. In the $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_x and $MPEG_{17}$-*b*-$PLLA_{160}$-*b*-PLL_x groups cell attachment was significantly enhanced. The cell attachment on triblock polymer surfaces was less influenced by the presence of serum compared with tissue culture polystyrene (TCPS surface) (Figure 3).

Triblock copolymer effected on cell spreading and cytoskeleton organization

SEM images of BMSCs cultured on the various polymer membranes are shown in Figure 4. After one hour incubation (Figure 4 A1-E1) on the polymer surfaces, the BMSCs were evenly spread and revealed a flatted appearance on the triblock

membranes (Figure 4 D1), similar to the cell morphology on TCP surface (Figure 4 E1). However, cells on PLLA (Figure 4 A1, A2) and diblock membranes including $MPEG_{17}$-*b*-$PLLA_{94}$ (Figure 4 B1) and $PLLA_{77}$-*b*-PLL_{72} (Figure 4 C1) appeared to be far less spread out and had less regular cell morphology; numerous filopodia were stretched out from the cells on these membranes (Figure 4 B1&C1). Generally, after 2 hours incubation (Figure 4 A2-E2) the cell morphology became flatter and showed more cytoplasmic extensions over the entire polymer surface compared to 1 hour incubation. Cells on triblock copolymer appeared to be more evenly spread with a flatten appearance compared to cells on PLLA or diblock copolymer surfaces (Figure.4).

To further assess cell spreading, the areas of individual BMSCs attached on different membranes at early time points (1 and 2 hours) were quantified with the aid of Image J software (http://rsb.info.nih.gov/ij/index.html). The cell areas were generally greater following two hours incubation compared to one hour incubation in all the observed cultures. It was noted that cells attached on the triblock copolymers were similar to TCPS and showed a larger area of spreading than those grown on the diblock films or PLLA polymers in both the one and two hour incubation (Figure 5).

Figure 6 shows the cellular expression of F-actin fibres on different copolymer surfaces. In the first hour cells cultured on $MPEG_{17}$-*b*-$PLLA_{94}$ PLL_{107} triblock copolymer (Figure 6 C1) and TCPS surface (Figure 6 D1) showed a spreading shape, exhibiting an abundant and well-organized cytoskeletal structure. The F-actin fibres

were arranged in a linear orientation in the cells on the triblock copolymer surface (Figure 6 C1). By contrast, most cells on PLLA (Figure 6 A1) and $MPEG_{17}$-*b*-$PLLA_{94}$ (Figure 6 B1) showed a round morphology and small size, and had fewer projections. F-actin was seen as ring-shaped dots distributed around the cell boundary or nuclei (Figure 6 A1, B1).

After 24 hours incubation the cells revealed an extended actin structure (Figure 6 A2-D2). On the triblock copolymer surface (Figure 6 C2), abundant actin filaments were clearly visible passing across the cell and over the nucleus, and individual actin bundles were easily distinguishable. In contrast, the F-actin filaments on the PLLA polymer film (Figure 6 A2) were sparsely distributed around the cell periphery. On the diblock polymer surfaces the F-actin displayed intense dots or broken lines (Figure 6 B2).

mRNA expression of ECM and adhesion molecule genes in initial attached BMSCs

The above results demonstrated that triblock copolymers not only enhanced BMSC attachment, but also regulated cell speading and cytoskeleton organization. To unveil the molecular mechanism regulating cell adhesion, the adhesion molecules, which play the imperative role in early cell attachment and spreading, were analysed in early attached BMSCs on PLLA, TCP, and $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107} surfaces. Figure 7 shows the results of fold changes of 84 adhesion genes in BMSCs attached on the surfaces between PLLA and TCPS (A), $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107} and TCPS (B),

and $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107} and PLLA (C). The volcano plots and tables displayed the genes which changed significantly ($p < 0.05$) (above the blue line) and which changed more than 3 folds (beyond the pink line). Compared with TCP surface, BMSCs attached on PLLA surface showed an overwhelming downregulation of most adhesion genes. Seven out of eight significantly regulated genes were down regulated, which included CDH1, THBS1, VCAM1, THBS2, ITGA1, Col16A1, and Col12A1. The only upregulated gene was ITGAM (Figure 7A and Table 1). However, BMSCs attached on the triblock surface showed a completely different gene regulation profile when compared with BMSCs attached on TCP surface. In five significantly regulated genes, four genes were upregulated including ITGA, ITGAM, LAMA, and MMP7. The only downregulation gene was THBS1 (Figure 7B and Table 2). Compared with PLLA surfaces, triblock copolymer surfaces showed a significant upregulation of adhesion genes. In seven significantly regulated genes, six genes were upregulated in BMSCs attached on triblock copolymer surfaces including CDH1, ITGL, LAMA3, NCAM1, SGCE, and COL16A1. The only down regulation gene was MMP1 (Figure 7C and Table 3).

DISCUSSION

Interactions between cells and polymers are mediated by proteins, which are secreted by the cells, immobilized on the biomaterial surface, or absorbed from *in vitro* culture medium or *in vivo* body fluids. To improve mesenchymal stem cell attachment on

PLLA surfaces, MPEG and PLL have been used to modify the PLLA surface to achieve a more hydrophilic surface. In this study triblock copolymers were synthesized to combine the property of PLL and PEG in the modification of PLLA to achieve optimized cell attachment and spreading. We further investigated and identified that modified polymer surfaces could directly influence the ECM and adhesion gene expression of BMSCs. This study for the first time reported that downregulation of ECM and adhesion genes in BMSCs were responsible for the impaired cell attachment on PLLA surface. Modification of PLLA with PEG and PLL increased the expression of ECM and adhesion molecule genes and consequently enhanced cell affinity.

MPEG has been widely used for polymer modification. In this study cell attachment on MPEG-*b*-$PLLA_x$ diblock copolymers was significantly influenced by the presence of serum in the culture medium. Similar report has been reported that endothelial cells, platelets and monocytes on diblock copolymer surfaces can be initially inhibited by the introduction of PEG [17]. PEG coatings have the ability of repelling protein adsorption and cell adhesion resistance [18]. However, it was found in this study that in serum-free conditions the reasonable content of PEG enhanced cell attachment. This may be due to the reasonable level of PEG incorporated in the polymer which produces well-solvated polymer brushes on the surface at biomaterial-aqueous solution layers [19-22], and which gives more degree of freedom for cell adhesion molecules by working as a spacer. It is known that low PEG concentrations on surfaces regulates the extent, as well as the quantity, conformation and specific

bioactivity of the adsorbed protein, which inhibits the onset and dynamics of cell adhesion and migration [23].

Poly ($_L$-lysine) (PLL) is a synthetic molecule used to enhance cell attachment to plastic and glass surfaces due to its hydrosolubility and functional NH_2 groups, which improves its affinity for proteins and cells [24]. In this study we observed that PLL played a significant role in enhancing initial cell attachment in both serum and serum-free conditions. The enhanced cell adhesion by poly($_L$-lysine) found in this study is due to the improved adhesive properties attributed to the PLL surface treatment, which alters the substrate surface charge from negative to positive [2, 12, 24, 25]. In addition to promoting cell adhesion, PLL also enhances the adsorption of serum or extracellular matrix proteins to the culture substrate; however, at a certain concentration of PLL its effect on cell attachment decreases. The reason for this is unclear, but a high density of adhesion ligands may induce a surface which is too hydrophilic to promote cell adhesion [26, 27].

MPEG-*b*-PLLA-*b*-PLL triblock copolymers were developed to minimize the interference from non-specific proteins and improve cell attachment quality. When the triblock copolymer was blended with PLLA, the PLLA segments of the MPEG-*b*-PLLA-*b*-PLL copolymers interact and entangle with the PLLA matrix to form a stable MPEG-*b*-PLLA-*b*-PLL layer on the PLLA surface [12]. The hydrophilic MPEG and PLL chains form an extended layer from the copolymer surfaces. Four groups of copolymer films were designed to investigate the influence of the copolymers on the initial attachment by varying the contents of MPEG, PLLA

and PLL and the chain length of these ligands. It was noted that, in both serum and serum-free conditions, cell attachment on triblock copolymer surfaces significantly improved compared to the diblock copolymer or PLLA alone. With increased PLL content an increased number of cells adhered on the triblock copolymer surfaces. However, the chain length of MPEG, PLLA and PLL also played an important role in regulating cell attachment.

In the series of activities taking place during early cell attachment, cells go through a variety of morphologies, from a small rounded shape, followed by a larger flattened appearance, to the natural stretched form. These changes are regulated by the ECM and cell adhesion molecules. In this study, the Triblock polymers were designed to regulate protein adsorption and cell adhesion. Cells on triblock copolymer surfaces exhibited a more spread out morphology than on PLLA and diblock copolymer surfaces and details of cell spreading and cytoskeletal organization varied on different copolymer surfaces. More well-organized actin was expressed in BMSCs on triblock copolymer and TCPS surfaces after 1 and 24 hours incubation compared to cells on either PLLA or diblock copolymer surfaces. This result suggests that triblock polymer surfaces provided more favourable conditions for initial BMSC attachment, via the spreading of actin cytoskeleton. Cytoskeletal microfilaments, in conjunction with microtubules and intermediate filaments, provide an architectural framework for cell spreading. In addition, microfilaments containing F-actin have been involved in several cellular activities, such as cytoplasmic streaming, cell division, cell shape change, cell surface events, and motility [28, 29]. The actin

cytoskeleton plays important roles in cell spreading and migrating, and is intimately related to processes such of cell-substrate adhesion.

To further understand the molecular mechanism of modified PLLA surfaces on cell adhesion, the 84 ECM and adhesion molecule genes were analysed. ECM and cell adhesion molecules are two groups of functional genes in cell affinity. It is known that upon contact with a substrate surface, cells began to secrete proteins including collagen, fibronectin, laminin, glycoproteins and other ECM. The secreted matrix is then used to support the adhesion of cells via adhesion receptors known as integrins. The integrin receptors have ligand binding domain and once this binding occurs, several key events are initiated, which affect cell signalling, nuclear organization and cytoskeletal formation. Therefore, understanding the effect of modified polymer surfaces on initial adhesion gene expression will eventually reveal the mechanism responsible for controlling the subsequent behaviour of BMSCs. In this study, the most significantly influenced cell adhesion genes in triblock modified PLLA were the upregulation of Cadherin 1 (8.1 fold) and Integrin L (5.3 fold) in comparison to PLLA surface, as well as Integrin L (8.6 fold) and Integrin M (4.4 fold) compared with TCPS. Cadherin is tri-functional molecules, associating with integrins, which bind ligands on other cells or in the extracellular matrix, connect to the cytoskeleton inside the cell, and regulate intracellular signaling pathways [30]. Integrins interact with the ECM through their extracellular domains, and with components of the cytoskeleton and also signaling molecules through their intracellular domains. Through these interactions, integrins can regulate many cellular functions, like cell

adhesion, motility, shape, growth, and differentiation [31]. After the binding of a ligand, the integrins will cluster together into focal contacts. This is an area of close contact between a cell and the ECM, consisting of additional cytoskeletal proteins, adapter molecules, and kinases. After clustering, cytoskeletal and signaling molecules will be recruited and activated [31]. Besides the recruitment of signaling molecules and signaling, integrin activation also results in changes in the organization of the cytoskeleton, subsequently affecting cell adhesion and mobility [32]. The upregulation of Cadherin and Integrin in Triblock modified PLLA surface explains the better cell attachment and cell spreading with well organised cytoskeleton. Interestingly BMSCs on PLLA surface Cadherin gene was significantly downregulated (5.36 fold) compared with BMSCs on TCPS. Therefore, modification of PLLA surface can significantly change Cadherin gene from downregulation to upregulation. Another interesting adhesion gene identified in this study is Thrombospondin (TSP), which was significantly down regulated in both PLLA and triblock modified PLLA surfaces compared with TCPS. TSP is designated a multifunctional protein involving the process of angiogenesis, apoptosis, activation of TGF-beta [33]. In relation to cell adhesion TSP promote cell-substratum adhesion of a variety of cells, including platelets, melanoma cells, muscle cells, endothelial cells, fibroblasts, and epithelial cells. The adhesion-promoting activity of TSP has been species independent, specific, and not due to fibronectin pathway [33]. Therefore, compared with tissue culture plate, BMSCs attachment on PLLA based materials is

regulated through a group of integrins and fibronectin mediated pathways, not via the specific TSP regulated cell-substratum adhesion.

CONCLUSIONS

MPEG-*b*-PLLA-*b*-PLL copolymers showed significantly improved initial BMSC attachment and spread via the organization of the cytoskeleton, which were the consequence of upregulation of Cadherin and Integrin genes in early attached BMSCs. This study proposed an interactive effect of modified PLLA copolymer of MPEG-*b*-PLLA-*b*-PLL on the attachment of BMSCs and has for the first time identified a group of critical ECM and adhesion genes that are responsible for the BMSC initial attachment on modified PLLA surfaces.

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Captions

Figure 1. BMSC attachment on surfaces of polymer films $MPEG_{17}$-*b*-$PLLA_x$ after incubation for 1 hr. Bars represent the relative ratio against control (PLLA). *: $P < 0.05$ against the control (PLLA)

Figure 2. BMSC attachment on surfaces of polymer films of $PLLA_x$-*b*-PLL_y after incubation for 1 hr. Bars represents the relative ratio against control (PLLA). *: $P < 0.05$, **: $P<0.01$ against the control (PLLA)

Figure 3. BMSC attachment on surfaces of different groups of polymer films after incubation for 1 hr. Bars represents the relative ratio against control (PLLA). A, B, C and D separately represent groups $MPEG_{17}$-*b*-$PLLA_{56}$-*b*-PLL_y, $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_y, $MPEG_{17}$-*b*-$PLLA_{153}$-*b*-PLL_y and $MPEG_{17}$-*b*-$PLLA_{160}$-*b*-PLL_y . *: $P < 0.05$, **: $P<0.01$ against the control (PLLA)

Figure4. Representative scanning electronic microscopy images of BMSCs cultured on polymer films and TCPS for a period of 1 h and 2 hrs in serum medium. Spheroid cells with filopodia on PLLA surface (A); large cells with larger filopodia on the copolymer surface of $MPEG_{17}$-*b*-$PLLA_{94}$ (B) and $PLLA_{77}$-*b*-PLL_{72} (C); flattened

morphology representative of well-spread cells with wide lamellipodia on the surface of $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107} (D) and TCPS (E).

Figure5. Areas of BMSCs from six random fields measure using ImageJ software. Data are expressed as BMSC area after seedinf for 1 and 2 hrs in serum medium. *: $P < 0.05$, **: $P<0.01$ against the control (PLLA)

Figure 6. Representative confocal laser scanning microscopy images of BMSCs cultured on polymer films and TCPS for endpoints 1 hr and 24 hrs in serum culture medium. Fluorescently-labelled actin is shown in green and fluorescently-labelled nuclei are shown in red. A1-D1 separately represents PLLA, $MPEG_{17}$-*b*-$PLLA_{94}$, $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107} and TCPS for 1 hr, A2- D2 for 24 hrs.

Figure 7. Gene expression volcano plots for PLLA/TCPS (A), $MPEG_{17}PLLA_{94}PLL_{107}$/PLLA (B) and $MPEG_{17}$-*b*-$PLLA_{94}$-*b*-PLL_{107}/TCPS (C). The volcano plots indicates a fold-changes in gene expression displaying the genes which changed significantly ($p < 0.05$) (above the blue line) and which changed more than 3 folds (beyond the pink line). The details of significant regulated genes were listed in the tables.

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