

Development of a novel three-dimensional biocompatible nanofibrous scaffold for the expansion and hepatogenic differentiation of human bone marrow mesenchymal stem cells

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Abstract

In this present study, we examined the differentiation potential of human bone marrow derived mesenchymal stem cells (hBMSCs) into hepatocytes on a three-dimensional (3D) nanofibrous scaffold formed by Poly (ϵ -caprolactone) (PCL), collagen and polyethersulfone (PES). The nanofiber was prepared by the electrospinning technique. HBMSCs were isolated using combining gradient density centrifugation with plastic adherence. Flow cytometric analysis was used to identify the isolated MSCs. The performance of the cells on the scaffold was evaluated by scanning electron microscopy (SEM) and MTT assay. The hBMSCs were then cultured in a hepatic differentiation medium containing hepatocyte growth factor (HGF), oncostatin M (OSM) and dexamethasone (DEX) for up to 21 days. The results showed that the isolated hBMSCs expressed specific markers such as CD44, CD166, CD105 and CD13. The integrity of the MSCs was further confirmed by their differentiation potential to osteogenic and adipogenic lineages. Scanning electron micrographs and MTT analysis revealed that the cells adhered and proliferated well on the nanofibrous hybrid scaffolds. Immunocytochemical analysis of albumin and α -fetoprotein (AFP) showed the accumulation of these markers in the differentiated cells on the

scaffold. Hepatocyte differentiation was further confirmed by showing expression of albumin, AFP and cytokeratin-19 (CK-19) at mRNA levels in differentiated cells. In conclusion, the evidences presented in this study show that the engineered scaffold is promising for maintenance of hepatocyte-like cells suitable for transplantation.

Keywords: Mesenchymal stem cells; Differentiation; Hepatocyte; Nanofiber; Scaffold.

INTRODUCTION

Tissue engineered liver is expected to become an effective treatment for patients suffering from hepatic failure (Lorenzini *et al.*, 2007; Kuling and Vacanti., 2004). Regenerative medicine and tissue engineering require two complementary key ingredients: 1) biologically compatible scaffolds that can be readily adopted by the body system without harm, and 2) suitable cells including various stem cells or primary cells that effectively replace the damaged tissues without adverse consequences. The scaffold should mimic the structure and biological function of the native extracellular matrix (ECM). A well known feature of the native ECM structures is the nanoscaled dimensions of their physical structure (Horii *et al.*, 2007; Hosseinkhani *et al.*, 2006).

Design of nanofibers is of important concern in the effective application of these nanostructured materials. For example, nanofibers containing drugs can perform controlled drug release (Hosseinkhani *et al.*, 2007, Zeng *et al.*, 2003; Kenawy *et al.*, 2002). In recent

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years, with respect to tissue engineering applications of nanofibers, a wide variety of nanofibrous scaffolds have been produced (Mohammadi *et al.*, 2007; Xin *et al.*, 2007; Hosseinkhani *et al.*, 2006; Shih *et al.*, 2006). Different techniques have been used for the formation of nanofibrous materials (Smith and Ma, 2004). There is an increasing interest towards the employing of electrospinning for scaffold fabrication because the mechanical, biological and kinetic properties of scaffold are easily manipulated by altering the composition of the polymer solution and the processing parameters (Chong *et al.*, 2007). Another advantage of using the electrospinning process is its ability to produce a non-woven nanofibrous structure, with morphological and architectural features similar to those of the natural ECM, capable of promoting favorable biological responses in the seeded cells (Li *et al.*, 2006; Li *et al.*, 2005; Li *et al.*, 2002).

Poly (ϵ -caprolactone) (PCL), an aliphatic polyester which is bioresorbable and biocompatible, is generally used in pharmaceutical products (Ng *et al.*, 2007). Experimental results have shown that synthetic biodegradable PCL supports cell growth. However, in order to enhance proliferation and encourage cell ingrowth into the scaffolds, the biologically inert PCL nanofibers need effective hybridization with bioactive molecules (Zhang *et al.*, 2005). It has been reported that electrospinning of PCL with collagen gives encouraging results in improving cell-scaffold interactions (Zhang *et al.*, 2005; Venugopal *et al.*, 2005). Likewise, polyethersulfone (PES) has many fascinating properties including favorable mechanical strength, thermal and chemical resistance, and excellent biocompatibility (Barzin *et al.*, 2007; Sluma *et al.*, 1993). Therefore, a polymer blend of PCL/collagen/PES can overcome the shortcomings of natural and synthetic polymers, resulting in a new biomaterial with good biocompatibility and improved mechanical, physical and chemical properties.

For successful tissue regeneration, the cells constituting the tissues to be regenerated are necessary. Considering the proliferation activity and differentiation potential of the cells, stem cells are practically promising. The interest in adult stem cells has particularly been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in preclinical and clinical studies (Henningson *et al.*, 2003). Among the adult stem cells, human bone marrow derived mesenchymal stem cells (hBMSCs) have great potential for liver tissue engineering. Differentiation of BMSCs into mesoderm cell lineages such as hepatocytes (Di Bonzo

et al., 2008; Snykers *et al.*, 2007; Lee *et al.*, 2004) is now well established. So far, natural matrices have been used in liver tissue engineering from stem cells (Baharvand *et al.*, 2007; Ong *et al.*, 2006; Schwartz *et al.*, 2002; Fiegel *et al.*, 2000). These natural polymers are suitable for cell interaction, however, the scaffolds fabricated purely from these molecules exhibit poor mechanical strength and are not easy to handle. Large batch-to-batch variations upon isolation from biological tissues, of natural scaffolds (Marler *et al.*, 1998).

In this study, to improve the efficacy of scaffolds in liver tissue engineering, a new matrix was fabricated by combining three different molecules forming a nanoscale structure. This PCL/collagen/PES nanofibrous network was used to investigate its capacity in supporting the differentiation of the hBMSCs into hepatocytes, by evaluating expression of liver markers including albumin, α -fetoprotein (AFP) and cytokeratin-19 (CK-19).

MATERIALS AND METHODS

Chemicals and reagents: Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), glutamine, antibiotics and trypsin-EDTA solution were obtained from Gibco BioCult (Paisley, Scotland, UK). Antibodies for flow cytometric assay were obtained from Dako (Denmark) and Oxford Biomedical Research (UK). RNA extraction kit, cDNA Synthesis Kit and the materials for PCR amplification were purchased from Qiagen (USA) and Fermentas (USA). Chambered coverglass was obtained from Nunc (USA). Hepatocyte growth factor (HGF), dexamethasone (DEX), oncostatin M (OSM), monoclonal mouse anti-human antibodies (for albumin and AFP), goat anti-mouse FITC-conjugated immunoglobulin G (IgG), collagen, PCL, PES, Alizarin red staining kit, oil red o-staining kit, cell growth determination kit (MTT kit) and other reagents were purchased from Sigma Chemical Co (USA). Human hepG2 hepatoma cell line were obtained from the Pasteur Institute of Iran (Tehran).

Isolation and culture of hBMSCs: Bone marrow aspirates (10 ml) were obtained from iliac crests of human donors (aged 19- 32 years) at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran. The bone marrow samples were obtained after the informed consent of the donors according to the guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran. Based on previous reports

(Majumdar *et al.*, 1998; Phinney 2002), mesenchymal stem cells were isolated from bone marrow samples as described below. The aspirates were diluted with phosphate buffer saline (PBS). The cell solution was gently overlaid on a Ficoll-Hypaque gradient (density, 1.077 g/ml) to eliminate unwanted cell types that were present in the marrow aspirate. Mononuclear cells were recovered from the gradient interface and washed with PBS after centrifugation at 400 g for 30 min at room temperature (RT).

The isolated mononuclear cell layers were then washed in PBS, resuspended in growth medium containing DMEM-low glucose supplemented with 15% (v/v) FBS, 2-mM glutamine, 100 μ g/ml of streptomycin, 100 U/ml of penicillin and plated in 75-cm² polystyrene plastic tissue-culture flasks. The cell cultures were maintained at 37°C in a humidified 5% CO₂ incubator (Galaxy, UK). Following 3 or 4 days of incubation, the non-adherent cells were washed away leaving behind the adherent cell population that was growing as fibroblastic cells in clusters. The hematopoietic stem cells and non-adherent cells were removed with every 3-4 days change in medium. When the cells reached 70-90% confluence, the cultures were harvested with 0.25% (w/v) trypsin-EDTA solution and plated in 25-cm² plastic cell culture flasks at a density of 10⁴ cells/cm².

Flow cytometric analysis of hBMSCs: The hBMSCs were detached from the tissue culture flasks after 14-33 days *in vitro* with trypsin/EDTA and counted. About 2×10⁵ cells were divided into aliquots and centrifuged at 1000 rpm for 5 min at RT. The pellet was resuspended in human serum and incubated for 30 min on ice. After centrifugation at 1000 rpm for 5 min, the pellet was resuspended in 3% (v/v) human serum albumin (HSA)/PBS and incubated with appropriate antibodies including Fluorescent isothiocyanate (FITC)-conjugated mouse anti-human CD44 (H-CAM), CD13, CD105 (Endoglin or SH2), CD34 and Phycoerythrin (PE)-conjugated CD166 (ALCAM), CD45 (leukocyte common antigen) for 1 hour on ice, washed twice in PBS and centrifuged for 5 min. The cells were resuspended in 100 μ l of PBS and studied by a Coulter Epics-XL flow cytometer (Beckman Coulter, CA). An isotype control with FITC- or PE-labeled antibodies was included in each experiment, and specific staining was measured from the cross point of the isotype using a specific antibody graph. The corresponding histograms were created by WinMDI 2.8 software (Scripps Institute, CA).

Fabrication and preparation of PCL/collagen/PES nanofiber scaffold: PCL was dissolved in chloroform to obtain a 10 wt% solution and type I collagen was dissolved in hexafluoroisopropanol (HFIP) at a concentration of 8% (w/v). PES was dissolved in an organic solvent mixture of N, N, dimethylformamide and tetrahydrofuran at a final concentration of 15 wt%. The electrospinning setup utilized in this study consisted of three syringes, a ground electrode (stainless steel drum, 3 and 5 mm outer diameters, 10 cm in length), and a high voltage supply. The distance between the tips and the rotating drum was in the range of 10-25 cm and the positive voltage applied to the polymer solutions was in the range of 15-30 kV. From the bulk material of the electrospun nanofiber mats, small discs with areas of approximately 0.8 cm² were cut out and placed in 24-well cell culture plates. All the scaffolds were soaked in 70 % filtrated ethanol for 2 hour. They were then incubated overnight in DMEM, at 37°C in a humidified 5% CO₂ incubator prior to cell seeding, in order to facilitate cell attachment onto the nanofibers.

Growth curve: The hBMSCs (1×10⁴ third passage cells/cm²) were transferred directly onto the scaffolds in 24-well plates and incubated for one hour. Thereafter, the scaffolds were soaked in DMEM-low glucose supplemented with 15% (v/v) FBS. Cell proliferation was evaluated during 12 days *via* 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, based on the cell growth determination protocol (Mosmann, 1983). Briefly, the culture medium was removed and replaced with 0.5 ml of medium without FBS; then, 25 μ l of MTT stock solution (5 mg/ml) was added to each culture being assayed. After 3 hour incubation, the medium was removed and the converted dye was solubilized with acidic anhydrous isopropanol (0.1 N HCl in absolute isopropanol). Absorbance of the converted dye was measured at a wavelength of 570 nm using ELISA reader (Labsystem Multiskan, Finland), with background subtraction at 670 nm. Data were obtained as the mean \pm standard error of mean of values from three cell seeded scaffolds. Statistical analysis was performed using Student's t-test (p < 0.05).

Osteogenic and adipogenic differentiation of hBMSCs: The potential of the isolated cells to differentiate into osteogenic and adipogenic lineages was examined. For osteogenic differentiation, the hBMSCs were induced for two weeks by Modified minimum essential medium (α -MEM) supplemented with 10%

(v/v) FBS, 0.1 μ M dexamethasone, 10 μ M β -glycerophosphate, and 50 μ M ascorbate-phosphate (Covas *et al.*, 2003; Kim *et al.*, 2004). The culture medium was changed twice a week for up to two weeks. The cells were fixed with methanol (90%) for 10 min at RT and identified by specific histochemical staining for calcium, using the Alizarin red staining kit. The stained material was examined with a phase contrast microscope (Nikon, Japan).

For adipogenesis, the cultured cells were incubated in the adipogenic medium DMEM supplemented with 10% (v/v) FBS, 1 μ M dexamethasone, 200 μ M indomethacin, 1.7 μ M insulin, 500 μ M isobutylmethylxanthine, 0.05 U/ml penicillin, and 0.05 μ g/ml streptomycin for two weeks. After 14 days, the cultured cells were detected for the presence of adipocyte, using the oil red o-staining procedure. Briefly, the cells were fixed in a 10% (v/v) solution of formaldehyde in aqueous phosphate buffer for 1 hour, washed with 60% isopropanol and stained with oil red o-solution for 10 min, followed by repeated washings with distilled water prior to destaining in 100% (v/v) isopropanol for 15 min (Janderova *et al.*, 2003; Ramriez *et al.*, 1992).

Hepatic differentiation protocol: The cultured hBMSCs at passage 3 (2×10^4 cells/cm²) were seeded onto the scaffolds. Hepatic differentiation was performed using a two-step protocol employing HGF, DEX and OSM, based on previous reports (Ong *et al.*, 2006; Lee *et al.*, 2004). Briefly, in the first step which lasted for seven days, the cells were cultured using a culture medium consisting of DMEM-low glucose supplemented with 15% (v/v) FBS, 20 ng/ml of HGF,

and 10^{-7} mol/l of DEX. In the second phase, OSM was added at a concentration of 10 ng/ml, during 21 days of differentiation. The culture media was changed twice weekly and hepatic differentiation was assessed by different experiments.

Scanning electron microscopy (SEM): The hBMSCs grown on the scaffolds were washed with PBS to remove non-adherent cells, fixed in 3% glutaraldehyde for 3 hour at RT and then dehydrated through a series of graded alcohol solutions. After drying, the scaffolds were mounted on aluminum stubs, sputter-coated with gold-palladium (AuPd) and viewed using SEM (Philips XL30, the netherlands).

Immunocytochemistry (ICC): After three weeks of differentiation, the cultured cells on the scaffold were harvested with 0.25% trypsin-EDTA solution and mounted on a chambered coverglass. After 24 hour of incubation at 37°C in a humidified 5% CO₂ incubator, the attached cells on the chambered coverglass were washed twice with PBS, fixed with 4% paraformaldehyde for 30-45 min at RT, and then permeabilized with 0.4% (v/v) Triton X-100 for 20 min. The washed cells were incubated overnight at 4°C with primary antibodies, including mouse anti-human albumin (1:1000) and mouse anti-human AFP (1:500). Subsequently, the cells were washed three times with PBS and incubated with fluorescence labeled second antibody and FITC-labeled goat anti-mouse IgG at 37°C, for 3 hour in the dark. After washing with PBS, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI); (1:1000) for the purpose of nuclear staining. The cells were visualized and photomicrographed using a fluorescence microscope (Nikon, TE-2000, Japan). The ratio of immunopositive cells to the total number of

Table 1. The primers and annealing temperatures used for RT-PCR.

Gene	Sequence	Product size (bp)	Annealing temperature (°C)
Albumin	F 5'-TGC TTG AAT GTG CTG ATG ACA GGG-3' R 5'-AAG GCA AGT CAG CAG GCA TCT CATC-3'	161	68
AFP	F 5'-TGC AGC CAA AGT GAA GAG GGA AGA-3' R 5'- CAT AGA CGA GCA GCC CAA AGA AGAA-3'	216	69
CK-19	F 5'-TGC GTG ACA TGC GAA GCC AAT-3' R 5'-ACC TCC CGG TTC AAT TCT TCA-3'	98	53
β -actin	5'- GTC CTC TCC CAA GTC CAC AC-3' R 5' - GGG AGA CCA AAA GCC TTC AT-3'	198	52

AFP: α -fetoprotein; CK-19: cytokeratin-19.

cell nuclei labeled with DAPI was recorded. Human hepG2 hepatoma cells cultured in 24-well plates were simultaneously stained for albumin and AFP and considered as positive control.

Reverse transcription polymerase chain reaction (RT-PCR): RT-PCR was used to determine the expression of albumin, AFP, CK-19 in the differentiated cells on the scaffolds. Briefly, total RNA was isolated from the cells using an RNA extraction kit (Qiagen, USA). Prior to reverse transcription, RNA samples

were digested with DNase I to remove contaminating genomic DNA. Standard reverse transcription was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). This involved 2 µg of total RNA and 0.5 µg of oligo(dt₁₈) per reaction, according to the manufacturer's instructions. PCRs were conducted using approximately 200 ng of cDNA to amplify a number of the hepatocyte marker genes listed in Table 1. After initial denaturation at 95°C for 1 min, PCR amplification was continued at a) 95°C for 40 s, b) annealing temperature for 40 s, and c) 72°C for

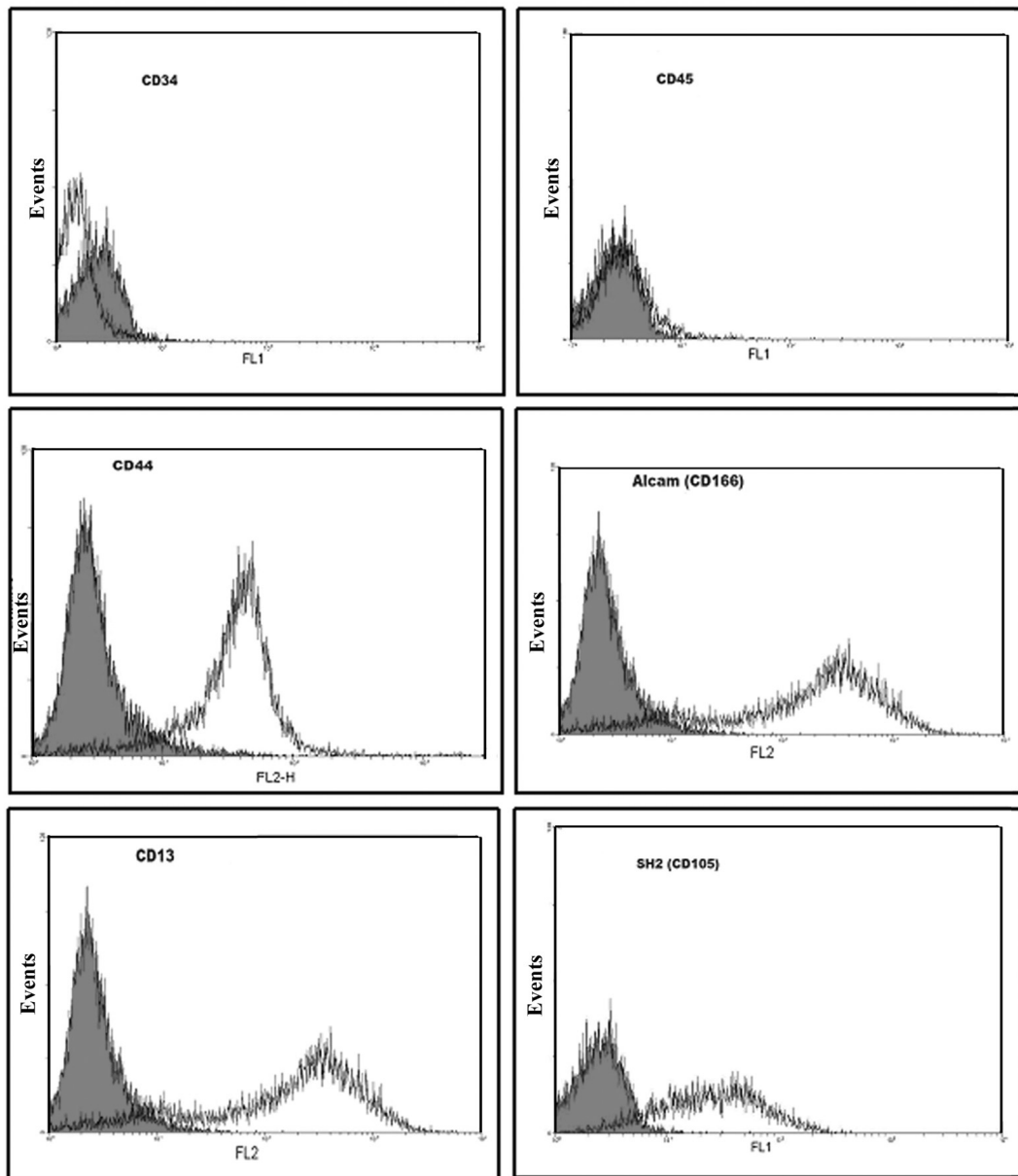


Figure 1. Flow cytometric analysis of the hBMSCs. Flow cytometric analysis was performed for the specific markers of MSCs and hematopoietic markers. MSCs were positive for CD44 (H-CAM), CD166 (Alcam), CD105 (Endoglin or SH2) and CD13. These cells were negative for CD34, and CD45. The shaded area shows the profile of the negative control.

1 min, for a total of 30 cycles, followed by a final extension at 72°C for 5 min. The amplified DNA fragments were electrophoresed on a 2% (w/v) agarose gel. The gels were stained with ethidium bromide (10 µg/ml) and photographed on a UV transilluminator (Uvidoc, UK).

RESULTS

Characterization of hBMSCs: The hBMSCs were cultivated from the mononuclear cell fraction of the bone marrow samples obtained from healthy donors. To ensure the removal of contaminating hematopoietic cells, the cells were selected based on plastic adherence and passaged three times prior to future use.

To ascertain that the culture-expanded cells were genuine MSCs, their phenotype and mesodermal differentiation potential upon exposure to mesenchymal-supportive conditions such as osteogenic and adipogenic-specific agents were examined. Flow cytometric analysis of the human BMSCs revealed expression of CD13, CD44, CD105, CD166, but not so for CD34 and CD45 (Fig. 1). Alizarin red staining and oil red o-staining confirmed the presence of calcium deposits, characteristic of osteogenic cells and lipid droplets in the differentiated cells, respectively, whereas undifferentiated MSCs were negative in both stainings (Fig. 2). In summary, these results indicate that the expanded cells have the basic properties of genuine MSCs.

Morphological observations: The scanning electron micrographs obtained prior to cell seeding revealed a

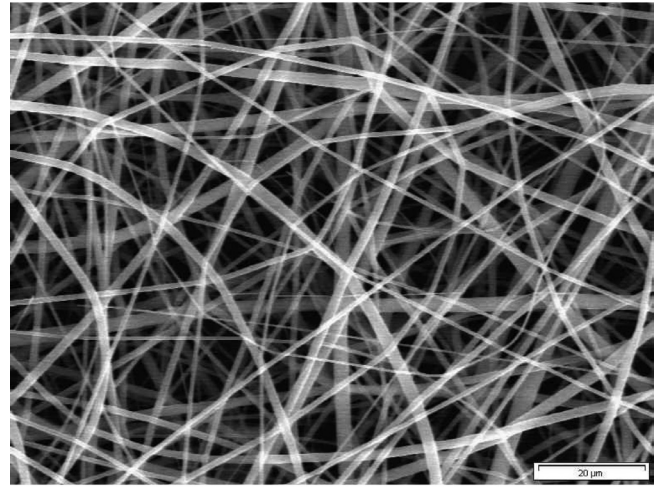


Figure 3. scanning electron microscopy (SEM) images of electro-spun PCL/collagen/PES nanofibers. The scaffold has a 3D, non-woven structure and consists of randomly oriented nanofibers. The average diameters of the pure PCL nanofibers were distributed in the range of 200-1500 nm. PES was distributed uniformly in the PCL nanofibrous structure. The region of distribution of PES and collagen ranged from 100 to 200 nm.

3D scaffold of non-woven, randomly oriented nanofibers. Figure 3 shows typical SEM micrographs of the hybrid matrix composed of PCL, collagen and PES nanofibers. As shown in this figure, PES was distributed uniformly in the PCL nanofibrous structure. The region of PES and collagen distribution ranged between 100 to 200 nm. However, the diameters of the pure PCL nanofibrous structures were broadly distributed in the range of 200-1500 nm. Furthermore, the produced hybrid matrix was approximately 500 µm in thickness. Figure 4 shows typical SEM micrographs of cells attached to the scaffolds prior to differentiation

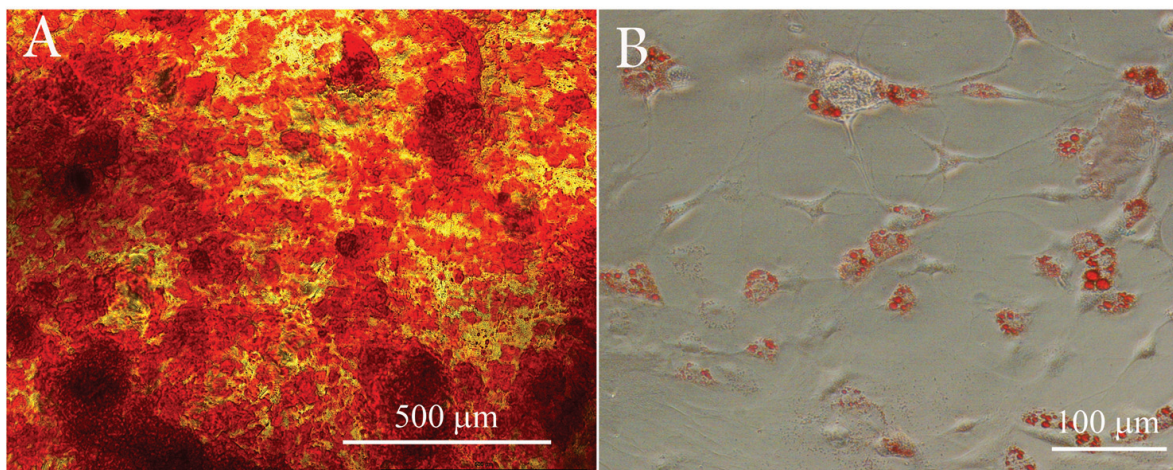


Figure 2. Differentiation of hBMSCs into adipocytes and osteoblasts. (A) alizarin red staining for osteogenic differentiation and (B) oil red-o-staining for adipogenic differentiation.

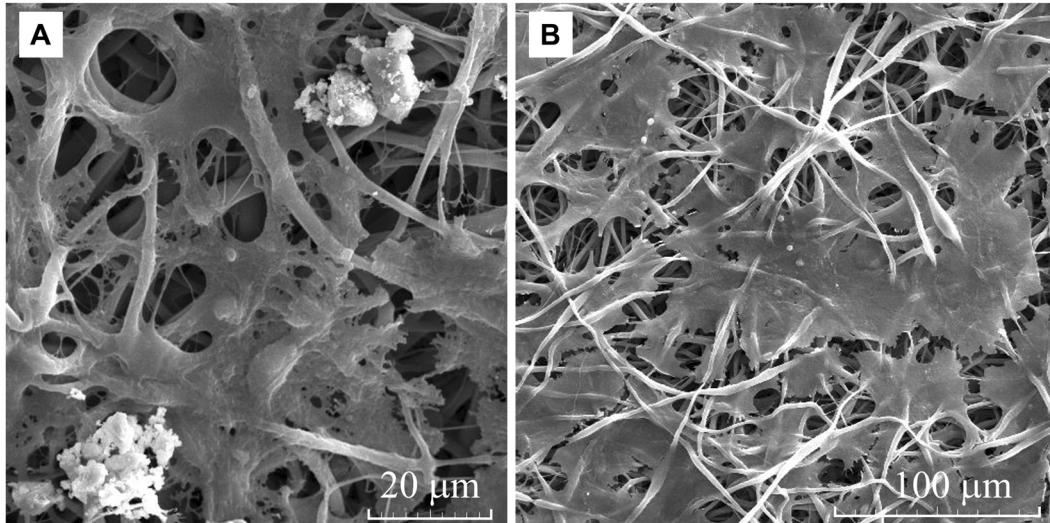


Figure 4. Scanning electron micrographs of the interaction between hBMSCs and the nanofiber scaffold. A) before differentiation, B) after differentiation.

and on day 21 of differentiation. As shown in this figure, the cells penetrated and adhered well onto the surfaces of the nanofibrous hybrid scaffolds. Proliferation profile of the hBMSCs during the 12 days of *in vitro* culturing is presented in figure 5. The obtained data showed that there was a progressive expansion of the attached cells on the scaffolds.

Immunocytochemical staining: To confirm *in vitro* hepatic differentiation of the hBMSCs on the scaffolds, expression of albumin (the most abundant protein synthesized by functional hepatocytes) and AFP (a protein indicative of hepatocyte morphology) was identified. The differentiated cells were stained positively for albumin and AFP on day 21 (Figs. 6 and 7). The expression of these protein markers was detected

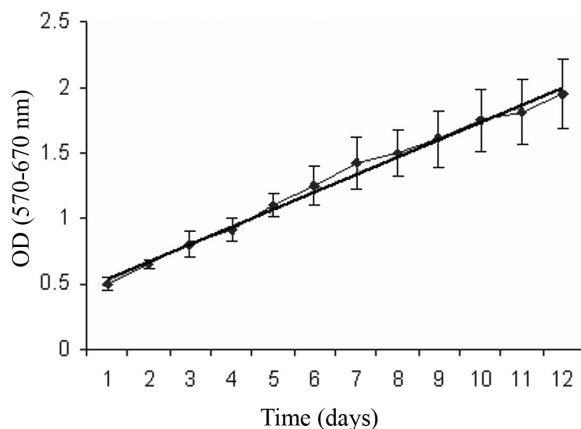


Figure 5. Growth curve of the hBMSCs cultured on the scaffolds. Data are mean \pm standard error of mean of three assays.

in human hepG2 hepatoma cells, used as positive control. The positive cells obtained from hBMSCs derived cells had large nuclei and polyhedral contours. Some of the positive cells were binuclear; an indication of hepatocyte maturation (Fig. 6A). The percentage of albumin and AFP positive cells were $47 \pm 4\%$, and $26 \pm 5\%$ in the differentiated cells, and $90 \pm 6\%$, and $88 \pm 10\%$ in hepG2, respectively. These markers were not expressed in MSCs used as the control group (data not shown).

Hepatic gene expression: Our findings confirmed hepatocyte generation by RT-PCR for the well known markers of hepatocyte differentiation such as albumin, AFP and CK-19. On day 0, the hBMSCs did not express mRNA of the hepatocyte lineage genes (Fig. 8), but, following the treatment, expression of the studied genes was detected in the induced cells on day 10 of differentiation. As shown in Figure 8, mRNA expression of the genes increased on day 21 of differentiation. The expression of β -actin, used as an internal control, was the same in both the undifferentiated and differentiated cells.

DISCUSSION

The hepatogenic differentiation of stem cells in natural matrices has been the subject of different reports (Baharvand *et al.*, 2007; Ong *et al.*, 2006; Schwart *et al.*, 2002; Fiegel *et al.*, 2000). The use of such natural scaffolds has been associated with some limitations.

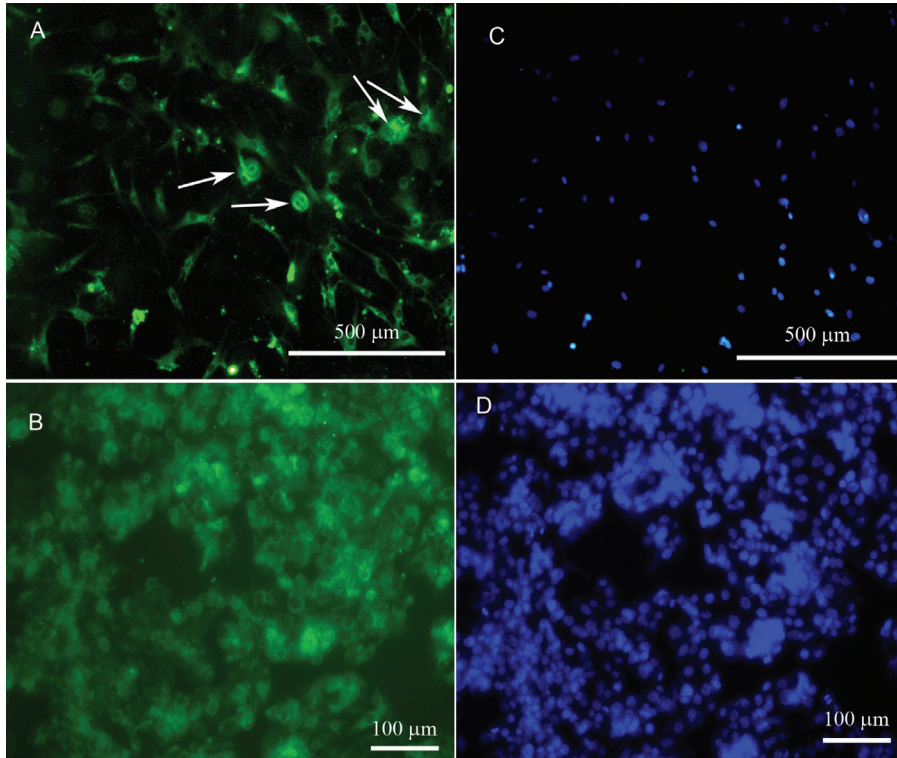


Figure 6. ICC staining of albumin. Positive staining of albumin was evidenced in the differentiated cells on day 21 of differentiation (A). The human hepatoma cell line (hepG2) was used as positive control (B). The cellular, the nuclei were stained with DAPI (C). for the differentiated cells, (D), the hepG2. Arrowheads show binucleated cells.

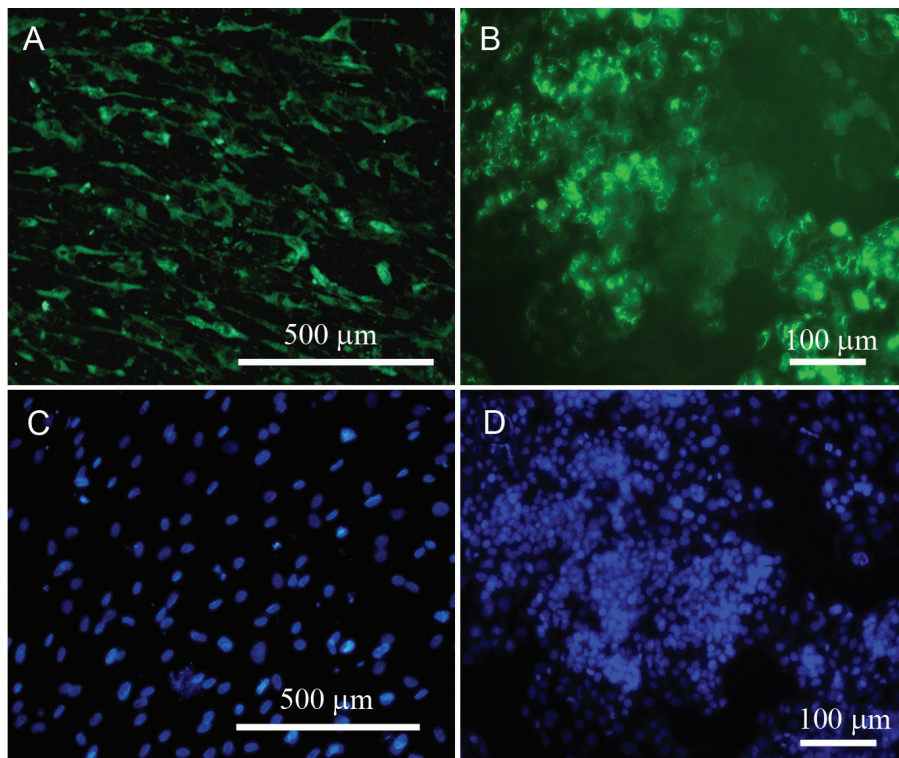


Figure 7. α -fetoprotein staining by ICC. Expression of this marker was visible on day 21 of differentiation (A). hepG2 was positively stained as positive control (B). Nucleus staining with DAPI had a significant signal (C). differentiated cells, (D), hepG2.

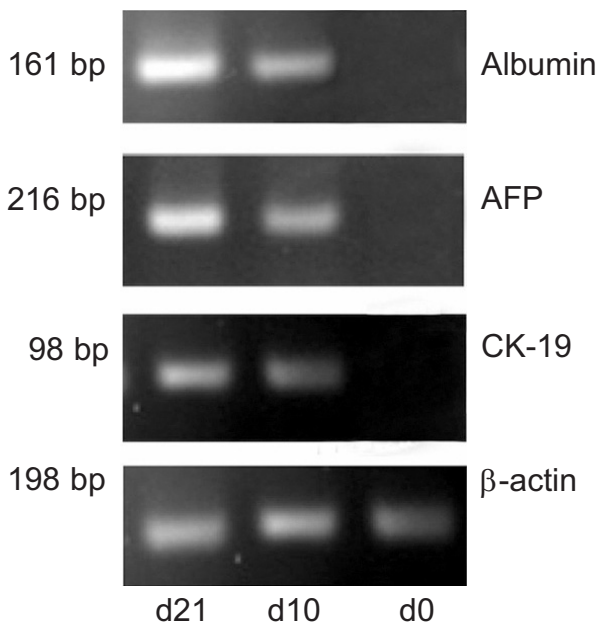


Figure 8. RT-PCR analysis of liver specific gene expression in hepatocyte-like cells on the scaffold. The expression of albumin, α -fetoprotein (AFP), cytokeratin-19 (CK-19) and β -actin (internal control) during *in vitro* differentiation is shown on days 10 and 21. The undifferentiated MSCs were used as control.

The problem with the control of pore size and porosity, large batch to batch variations upon isolation from biological tissues and poor biomechanical strength are major concerns.

In recent years, different synthetic scaffolds have been fabricated and used for liver tissue engineering (Ju *et al.*, 2007; Zavana *et al.*, 2005; Kulig K and Vacanti J, 2004; Semino *et al.*, 2004; Jian *et al.*, 2002; Torok *et al.*, 2001; Glicklis *et al.*, 2000). Although there is a significant interest in the use of nanofibers in tissue engineering from stem cells, but no report is available on the transdifferentiation of stem cells into the hepatic lineage, in an ECM-like nanofibrous configuration. The scaffold introduced in the present study is probably useful as a delivery vehicle for hepatocyte transplantation. Our findings show that the PCL/collagen/PES mesh allow the hBMSCs to adhere, proliferate and organize into hepatocyte-like cells.

Previous studies showed that nanoscaled features influenced cell behaviors (Rosenberg *et al.*, 1963). It has been demonstrated that cell adhesion and proliferation increased with decreasing fiber diameter ranging from 10 μm to 500 nm (Tian *et al.*, 2008). Since, the pore size of the engineered hybrid polymer is within the range of 200-1500 nm (Fig. 3), a nanofiber environment is thus created, which permits slow diffusion

of small molecules, metabolites and macromolecules such as gases, nutrients and growth factors. This feature of the polymer together with its non-woven architecture (Fig. 3) gives an ECM-like structure to this scaffold.

As described in the Methods section, the MSCs derived from bone marrow were first characterized and then transferred into the ECM-like nanofibers. Transdifferentiation ability of the isolated cells to osteogenic and adipogenic lineages was the first evidences to approve the nature of MSCs (Fig. 2). Expression of the markers for MSCs such as CD44, CD166, CD105, and CD13 (Haynesworth *et al.*, 1992) was also used to characterize the cells. Negative expression of certain markers such as CD34 and CD45, known as hematopoietic lineage markers, was used to confirm the purity of the MSCs (Fig. 1).

The results of cell attachment and proliferation (Figs. 4 and 5) revealed that this highly porous network is sufficient for the survival of cells for extended periods of time. The artificial scaffolds formed by the PCL/collagen/PES blend not only provided a suitable support for proliferation, but also allowed the cells to maintain their stability during differentiation (Fig. 4). It seems that collagen, as a major natural component of the scaffold, plays a major role in the favorable response of hBMSCs. The cells are not prominent in pure PCL nanofibers because of the absence of amino groups and hydrophilicity (data not shown). The favorable response of hBMSCs on the scaffold suggests the effective presence of collagen biomacromolecules.

Expression of biochemical markers, particularly albumin and AFP in differentiated hepatocytes are commonly used to identify the performance of stem cell derived cells (Thorgeirsson, 1996; Brill *et al.*, 1993; Shiojiri *et al.*, 1991). In the present study, albumin as the late marker of hepatic differentiation was found to be expressed in cells on day 21 of differentiation (Fig. 6A). Moreover, albumin-specific mRNA which was gradually expressed during the differentiation of cells on the scaffold, further attests to the functionality of hepatocyte-like cells (Fig. 8).

Another marker was AFP, that was found to be expressed at protein and mRNA levels in the differentiated cells (Fig. 7A and Fig. 8). The expression of AFP during the differentiation process suggests the presence of hepatic progenitors such as oval-shaped cells. The up-regulation of CK-19 at the mRNA level as one of the liver markers during differentiation (Thorgeirsson, 1996) shows the development of MSCs into hepatocyte-like cells in this study (Fig. 8).

The evidences presented in this paper clearly show the scaffold can support proliferation and hepatogenic differentiation of hBMSCs. However, very little is known about the stimulating effects of this hybrid polymer on hepatic differentiation.

In summary, we have developed a 3D biocompatible nanofiber network useful in liver tissue engineering that may be a suitable delivery vehicle for transplantation and promising for the development of a bioartificial liver system. However, further experiments especially *in vivo* studies are required to approve the applicability of such nanofibers in liver tissue engineering.

Acknowledgments

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