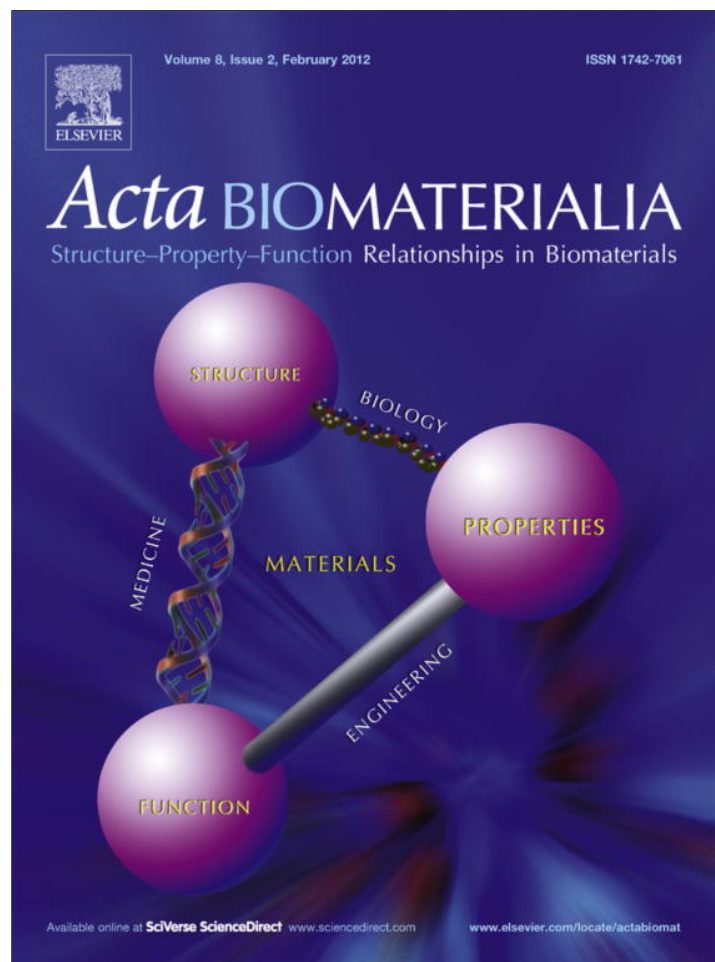


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Controlled release of bone morphogenetic protein 2 and dexamethasone loaded in core–shell PLLACL–collagen fibers for use in bone tissue engineering

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ABSTRACT

Electrospun nanofibers mimic the native extracellular matrix of bone and have generated considerable interest in bone tissue regeneration. The aim of this study was to fabricate novel poly(L-lactide-co-caprolactone) (PLLACL), PLLACL/collagen nanofibers blended with bone morphogenetic protein 2 (BMP2) and dexamethasone (DEX) for controlled release during bone tissue engineering (BTE). The morphology, surface hydrophilicity, and mechanical properties of the PLLACL/collagen nanofibrous mats were analyzed by scanning electron microscopy and water contact angle and mechanical stability determination. The performance of the scaffolds was investigated in terms of the viability and morphology of human mesenchymal stromal cells (hMSC) on the nanofibrous mats. BMP2 and DEX were successfully incorporated into PLLACL/collagen nanofibers by means of blending or coaxial electrospinning and the PLLACL/collagen blended fibers proved useful for hMSC culture. Release of the two growth factors from PLLACL/collagen nanofibrous mats in vitro was investigated by UV spectrophotometry. The release profiles for core–shell nanofibers showed more controlled release of the growth factors compared with the blended electrospun fibers. The experimental results show that controlled release of BMP2 and DEX can induce hMSC to differentiate into osteogenic cells for bone tissue engineering. The results imply that PLLACL/collagen nanofibers encapsulating two drugs and/or proteins have great potential in bone tissue engineering.

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1. Introduction

The number of bone graft procedures has been estimated to be over 1.5 million in the USA every year, making bone second only to blood on the list of transplanted materials [1]. However, because of the limited supply and associated donor site morbidity, and the risk of immune reactions to auto- and allografts, demand cannot be met [2–5]. With an increasing demand for and decreasing supply of traditional bone graft tissue, tissue engineering techniques are being developed to provide alternatives with properties

appropriate to clinical use. Electrospun polymer nanofibers have potential applications as drug release systems and to protect the activity of encapsulated drugs or proteins for tissue engineering applications [6]. Electrospun nanofibers have several advantages over other dosage forms, including the drug release profile, which can be finely tailored by modulation of the morphology, porosity and composition of the nanofiber membrane [7–10]; the small diameter of nanofibers with a high surface area is helpful for mass transfer and efficient drug release. For drug delivery systems there is particular interest in producing biodegradable nanofibers which could encapsulate and release drugs or biological growth factors over a long period of time [11]. The drug release characteristics depend on the drug encapsulated inside the resulting nanofibers. However, low drug delivery efficiency and burst release are some of the problems with these systems, therefore core–shell structured nanofibers have been developed to overcome the problem of burst release. Moreover, core–shell nanofibers can protect

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unstable biological agents from a harsh environment, deliver the bioactive molecules or drugs in a sustained way, and functionalize the surface of nanostructures without affecting the core material.

In recent times combined therapy with drugs and growth factors with different therapeutic effects have been shown to be an effective way treating disease and promoting tissue regeneration [12,13]. In order to optimize their effects different drugs and growth factors should be used at optimal dosages for different periods. One of the main challenges of combined therapy is to control the release behavior of each drug independently. However, simple drug delivery systems cannot fulfill the needs of such therapies for tissue engineering. Therefore, fabrication of multi-drug delivery systems which can control the release behavior of each drug is desired. Su et al. developed a dual drug loaded system by emulsion electrospinning, which simultaneously contains drugs in the core and outer layer of the nanofibers. The resulting dual drug loaded nanofibers show different release profiles [14]. Bone tissue is a mineralized connective tissue formed by cells, an organic matrix (type I collagen fibers) and inorganic salts (hydroxyapatite (HA)). Several researchers have focused on electrospun nanofibers for application in bone tissue engineering, such as HA/chitosan [15], poly(lactic-co-glycolic acid)/collagen/HA [16], poly-L-lactic acid/collagen/HA [17] and polycaprolactone/collagen/HA [18], to biomimic the components of the extracellular matrix (ECM) using electrospun nanofibers to promote osteoblast proliferation.

Mesenchymal stromal cells (MSC) are an adult stromal cell having the ability to differentiate into various mesodermal cell lineages, such as osteoblasts, endothelial cells, chondrocytes, etc. They can exert a profound immunosuppressive effect via modulation of both cellular and innate immune pathways. Their ability to be readily isolated from a number of tissues and expanded in vitro makes them attractive candidates for systemic immunosuppressive therapies [19,20]. BMP2 can induce differentiation of MSC into osteogenic cells and promote osteoblast proliferation [21,22]. DEX is a synthetic glucocorticoid which has been proved to support the osteogenic differentiation of hMSC in vitro, together with β -glycerophosphate and ascorbic acid [23,24]. Incorporation of both BMP2 and DEX into a biomaterial matrix may improve their clinical efficacy in bone tissue engineering. Moreover, to maintain their effective concentration, prolong their availability and reduce the systemic risk of high burst doses, controlled release of growth factors from the biomaterial scaffolds is essential in bone tissue engineering [25–28].

Osteoinductivity and osteoconductivity are very important for bone tissue scaffolds. In our present study we have designed a dual drug and/or protein loaded core-shell scaffold for bone tissue engineering. Two bioactive agents, DEX and BMP2, were incorporated into PLLACL/collagen nanofibers by coaxial electrospinning. The objective of this study was to fabricate novel core-shell nanofibers having the capability to encapsulate DEX and BMP2 to control/alter the drug release profile and to promote osteogenic expression by hMSC for bone tissue engineering.

2. Materials and methods

2.1. Materials

Poly(L-lactide-co-caprolactone) (PLLACL) with a molar ratio of 75% L-lactide was purchased from Sigma-Aldrich Co. (Milwaukee, WI). Collagen type I (molecular weight $0.8-1 \times 10^5$ Da) was purchased from Sichuan Ming-rang Bio-Tech Co. Ltd., China. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP), DEX and bovine serum albumin (BSA) was purchased from Sigma-Aldrich Co. (St Louis, MO). BMP2 (human) was bought from Prospec (Rehovot, Israel).

2.2. Preparation of electrospun PLLACL/collagen nanofibers and core/shell PLLACL/collagen nanofibers loaded with BMP2 and DEX

PLLACL was dissolved in HFP at 10% w/v and magnetically stirred at room temperature overnight. To fabricate the PLLACL/collagen blended nanofibers PLLACL and collagen powders were mixed and dissolved in HFP at a ratio of 1:1 and 3:1 (w/w) overnight at room temperature at a concentration of 10%. The solution was then loaded into a syringe with a spinneret needle (27G1/2) and a high voltage power supply (Gamma High Voltage Research, Ormond Beach, FL) was applied to fabricate nanofibers. The solvent evaporates during the electrospinning process and the resultant nanofibers were vacuum dried overnight to remove any residual solvent.

PLLACL-collagen 3:1 was chosen as the bone tissue engineering scaffold material in this study. The concentration of PLLACL-collagen in HFP solution was 10%, and the concentrations of DEX, BMP2 and BSA in phosphate-buffered saline (PBS) were $100 \mu\text{g ml}^{-1}$ (the maximum solubility of DEX in PBS is $140 \mu\text{g ml}^{-1}$), $25 \mu\text{g ml}^{-1}$ and 1 mg ml^{-1} , respectively. Solution "A" was prepared by dissolving PLLACL/collagen in HFP and stirring overnight; DEX and BMP2 (stabilized by BSA) were dissolved in PBS to give a uniform solution. 20 min before electrospinning 1 ml of DEX + BMP2 solution was added to 10 ml of PLLACL-collagen. The mixture was stirred to obtain a uniform solution. Solution "B" was prepared using DEX and PLLACL/collagen in HFP as the shell solution, with BMP2 dissolved in PBS as the core solution. Solution "C" was fabricated by dissolving DEX and BMP2 in PBS to prepare the core solution, with PLLACL/collagen dissolved in HFP as the shell solution. In the resultant nanofibers the concentrations of DEX and BSA were 0.1 wt.% with 1 wt.% PLLACL-collagen, and the concentration of BMP2 was $25 \mu\text{g g}^{-1}$ PLLACL/collagen.

The details of electrospun solutions are shown in Table 1.

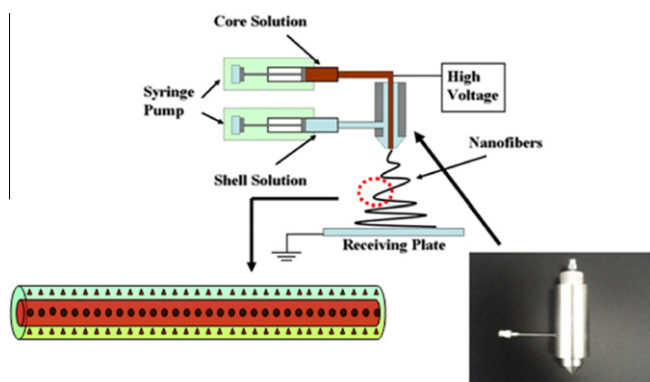
The equipment set-up comprises a syringe-like apparatus with an inner needle coaxially placed inside an outer one, as shown in Scheme 1. Two syringe pumps (Cole-Parmer Instrument Co., Vernon Hills, IL) were used to push the solutions from the inner and outer needles, respectively. The inner needle has an inner diameter of 0.8 mm and an outer diameter of 1 mm; the outer needle has an inner diameter of 1.8 mm. A copper electrode connects the inner needle directly to a high voltage supply. The electric potential transferred to the shell solution depends on the conductivity of the needle, the core and the shell solution. An aluminum foil was connected as a ground and then used to collect the fibers. The core solution was injected at a controlled flow rate of 0.10 ml h^{-1} and the shell solution at 1.0 ml h^{-1} . The distance between the needles and the collector was set at 15 cm. Fabricated electrospun nanofibers were kept at an ambient temperature of 22–25 °C and a relative humidity of 40–60% [6,8].

2.3. Characterization of the PLLACL/collagen nanofibers

A JEOL JSM-5600 LV digital vacuum scanning electron microscope was employed to examine the morphologies of the prepared PLLACL-collagen nanofibrous mats and DEX and BMP2 loaded nanofibrous mats. Prior to scanning electron microscopy (SEM) examination the specimens were sputter-coated with gold to avoid charge accumulation. Fluorescein isothiocyanate conjugated BSA (FITC-BSA) was used as the stabilizer instead of BSA to study BMP2 distribution in the core of the PLLACL-collagen nanofibers, by observing the distribution of FITC-BSA in the resultant fibers by laser scanning confocal microscopy (LSCM). Verification of the core-shell structure was by transmission electron microscopy (TEM) (H-800, Hitachi) at 100 keV. The samples for TEM observation were prepared by collecting the nanofibers on carbon-coated Cu grids.

Table 1
Components of the solutions for electrospinning.

Mats	Shell	Core	Type of nanofibers
A	PLLACL–collagen/ DEX + BMP2	PLLACL–collagen/ DEX + BMP2	Blended
B	PLLACL–collagen/DEX	BMP2	Core–shell
C	PLLACL–collagen	DEX + BMP2	Core–shell



Scheme 1. Basic set-up for coaxial electrospinning. The inset shows the special apparatus used in coaxial electrospinning.

Water contact angles for the nanofibrous mats were measured using a contact angle analyzer manufactured by the Data Physics Corp. (San Jose, CA). The water contact angle helps to identify the effect of drug location on the hydrophilicity of electrospun nanofibrous mats. During the measurements samples of the nanofibrous mats were first cut into 1 cm² square pieces and then placed on a testing plate. Subsequently, 0.03 ml of distilled water was carefully dropped onto the prepared specimens. The contact angles between water droplets and the nanofibrous mats were measured and recorded using a video monitor.

The tensile testing of samples (30 × 10 mm) was performed using a universal materials tester (H5 K-S, Hounsfield, UK) at an ambient temperature of 20 °C and humidity of 65%. A cross-head speed of 10 mm min⁻¹ was used for all specimens tested.

2.4. Proliferation and morphology of hMSC on PLLACL/collagen nanofibers

2.4.1. Cell expansion and seeding on nanofibers

hMSC (Lonza Walkersville Inc., Walkersville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with antibiotics in 75 cm² cell culture flasks. The cells were cultured until passage 4, and then they were harvested from third passage cultures by trypsin–EDTA treatment and replated. For cell seeding nanofibrous mats (round glass coverslips 15 mm in diameter) were sterilized under UV light for 2 h and washed with PBS three times. The hMSC were seeded at a cell concentration of 2 × 10⁴ cells cm⁻² on electrospun nanofibrous scaffolds and tissue culture polystyrene (TCP) as a control in 24-well plates.

2.4.2. Cell proliferation

The cell proliferation were quantified by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) using the CellTiter 96[®] Aqueous Assay kit (Promega, Madison, WI) after seeding for 2, 7 and 14 days. In this assay metabolically active cells break down the tetrazolium

salt in the MTS reagent to produce a soluble formazan dye, which can be observed at 490 nm in a spectrophotometric plate reader.

2.4.3. Cell phenotypes observation by immunocytochemistry

For the cell morphology studies an immunocytochemical staining technique was employed. hMSC cultured on nanofibers were washed with PBS to remove non-adherent cells and then fixed in 4% paraformaldehyde for 30 min at room temperature, followed by rinsing with PBS three times (15 min each time). Subsequently the cells were incubated with the primary antibody (Mouse monoclonal anti-actin, clone C4, Millipore, Billerica, MA) overnight at 4 °C. After washing with PBS, the samples were incubated for 1 h with the secondary antibody (FITC-conjugated goat anti-mouse IgG, Sigma–Aldrich Corp., St Louis, MO). Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole, dilactate (DAPI, Invitrogen Corp., Carlsbad, CA) for 30 min. The mounted samples were observed and viewed by LSCM.

2.5. Controlled drug release

In the controlled drug release study FITC–BSA was used as the stabilizer instead of BSA during electrospinning. Composite fibrous mats electrospun from solutions A, B and C, each weighing about 40 mg (encapsulating about 40 µg DEX and 400 µg BSA), were soaked in 20 ml of PBS in glass vials. The fibrous mats were incubated at 37 °C in the presence of 5% carbon dioxide. At various points of time 2 ml of the supernatant was removed from the vial, and replaced by an equal volume of fresh medium. The concentrations of FITC–BSA (at an optical wavelength of 458 nm) and DEX (at an optical wavelength of 242 nm) in the supernatant were then determined using a UV–vis spectrophotometer (WFZ UV-2102 Unique Technology, Shanghai, China). All processes were carried out in a dark room [29].

2.6. Seeding and differentiation of hMSC

PLLACL–collagen nanofibrous mats loaded with DEX and BMP2 were collected on round coverslips (15 mm diameter), and sterilized by UV irradiation for 1 h on each side. hMSC at passage 4 were harvested, then seeded onto the PLLACL–collagen nanofibers loaded with the two growth factors at a density of 1 × 10⁴ cells per scaffold. Nanofibers without growth factors and TCP were used as controls. The cell–nanofiber constructs were cultured in osteogenic differentiation medium (DMEM supplemented with 50 µg ml⁻¹ ascorbic acid and 10 mM β-glycerophosphate). The control cell–TCP constructs were cultured in standard osteogenic differentiation medium (DMEM supplemented with 50 µg ml⁻¹ ascorbic acid, 10 mM β-glycerophosphate, 10⁻⁷ M DEX and 50 ng ml⁻¹ BMP2) [25,30].

2.7. Alkaline phosphatase (ALP) activity

ALP activity was assessed by p-nitrophenol assay on days 3, 7, 14 and 21. 400 µl of p-nitrophenyl phosphate disodium salt (PNPP) solution (Phosphatase Substrate Kit, Pierce Biotechnology, Rockford, IL) was added to each sample and incubated at room temperature for 30 min. The reaction was terminated by adding 200 µl of 2 M NaOH. The absorbance was read at a wavelength of 405 nm. Standard curves used to determine the ALP concentrations produced by hMSC were constructed using an ALP standard (AnaSpec, San Jose, CA).

2.8. Immunocytochemical analysis

Nanofibrous mats with cells were fixed in 4% formaldehyde (Lancaster Synthesis Inc., Windham, NH) for 30 min after 14 days culture. After washing with PBS the nanofibers–hMSC were blocked

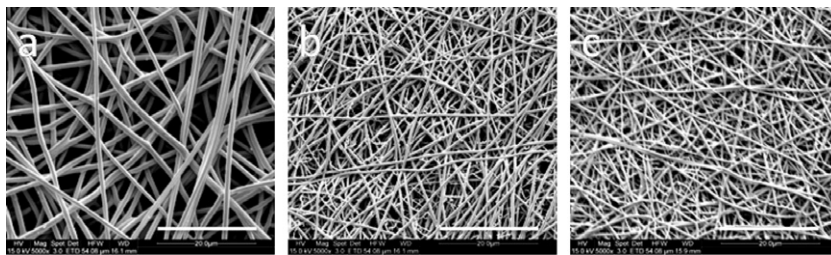


Fig. 1. Representative morphologies of electrospun nanofibers. (a) PLLACL (as a control); (b) PLLACL-collagen 3:1; (c) PLLACL-collagen 1:1. Scale bar 20 μm .

with 2% BSA for 1 h. Subsequently they were incubated with the primary antibody mouse monoclonal anti-osteocalcin (Invitrogen Corp., Carlsbad, CA) overnight at 4 $^{\circ}\text{C}$. After washing with PBS the samples were incubated for 1 h with the secondary antibody FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich Corp., St Louis, MO). Finally, the cells were counterstained with DAPI (Invitrogen Corp., Carlsbad, CA) for 30 min. The samples were observed and viewed by LSCM (Olympus Fluoview FV1000, Olympus Corp., Center Valley, PA).

2.9. Statistical analysis

Statistical analyses were performed using Origin 7.5 (OriginLab, Northampton, MA). Values (at least triplicate) were averaged and are expressed as means \pm SD. Statistical differences were determined by one-way ANOVA and differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Morphology and characterization of electrospun PLLACL/collagen fibers

PLLACL and PLLACL-collagen (3:1, 1:1) nanofibers were fabricated by electrospinning and are shown in Fig. 1. They are continuous, uniform and smooth, with interconnected pores (space) between the nanofibers. However, the fibers made from PLLACL (average diameter 859.3 nm) are much thicker than the PLLACL-collagen fibers (336.8 and 365.4 nm). One possible explanation for this is that the conductivity of the electrospinning solutions increases on the addition of protein [31].

The hydrophilicity of nanofibrous mats plays a pivotal role in determining the overall performance for tissue engineering applications. The water contact angle technique was used to test the hydrophilicity of the nanofibrous mats [32–34]. For pure PLLACL nanofibers the contact angle was $131.3 \pm 1.55^{\circ}$, which is much higher than that for blended collagen nanofibers (PLLACL-collagen 3:1, $40.4 \pm 2.3^{\circ}$) and (PLLACL-collagen 1:1, $29.75 \pm 1.68^{\circ}$). Blended

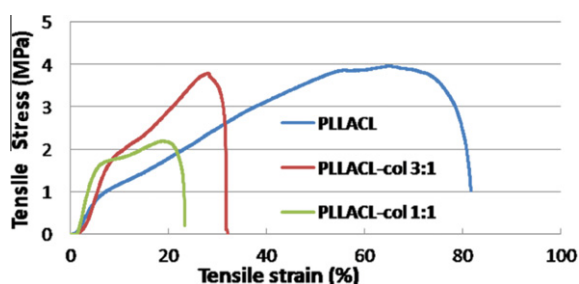


Fig. 2. Stress-strain curves for electrospun pure PLLACL nanofibers (blue), PLLACL-collagen 3:1 (red) and PLLACL-collagen 1:1 (green).

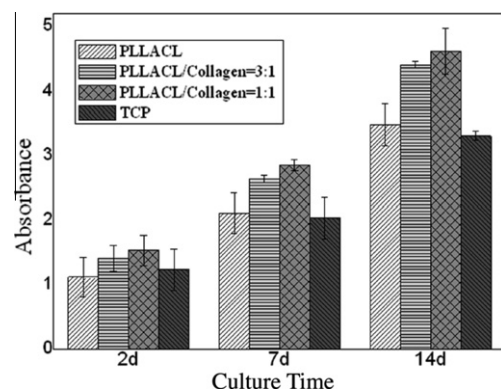


Fig. 3. hMSC proliferation on electrospun PLLACL, PLLACL-collagen 3:1, PLLACL-collagen 1:1 and TCP.

collagen nanofibers are hydrophilic and may enhance cell adhesion and cell proliferation [32].

The mechanical properties of nanofibers are important for successful application in bone tissue engineering. The average thicknesses of the PLLACL, PLLACL-collagen 1:1 and PLLACL-collagen 3:1 mats are 0.0242, 0.0259 and 0.0247 mm, respectively. Fig. 2 shows the stress-strain curves for PLLACL, PLLACL-collagen 3:1 and PLLACL-collagen 1:1 nanofibers under tensile loading. The ultimate tensile strength of the PLLACL nanofibrous mat was 3.96 ± 0.46 MPa, which is higher than that of the blended collagen electrospun nanofibrous mats (3.79 ± 0.31 and 2.19 ± 0.28 MPa). The strain at the ultimate tensile strength of the blended collagen nanofibers was significantly lower than that of pure PLLACL nanofibers [31,32]. Based on the SEM micrographs there had been little or no entanglement of the fibers. As such, the authors think that entanglement of the fibers is unlikely to contribute to their tensile strength.

3.2. hMSC proliferation and phenotype

The hMSC proliferation results on days 2, 7 and 14 determined by MTS assay are shown in Fig. 3. The results show that PLLACL-collagen nanofibers are more conducive to cell duplication compared with pure PLLACL and TCP on days 7 and 14 of culture.

The morphology and proliferation of hMSC on TCP, PLLACL and PLLACL-collagen are shown in Fig. 4. Fig. 4a–d shows that the cells had grown and were well spread on the nanofibers on day 7. However, the numbers of cells on the TCP and PLLACL nanofibers were less than on the PLLACL-collagen nanofibers. On day 14 the density of hMSC was very high on all the substrates, especially on collagen-based nanofibers. Based on the results for TCP vs. the results for the nanofibers it can be seen that a high porosity and a fibrous structure is favorable for cell adhesion and expansion. From the results it can also be seen that cell proliferation and expansion were more

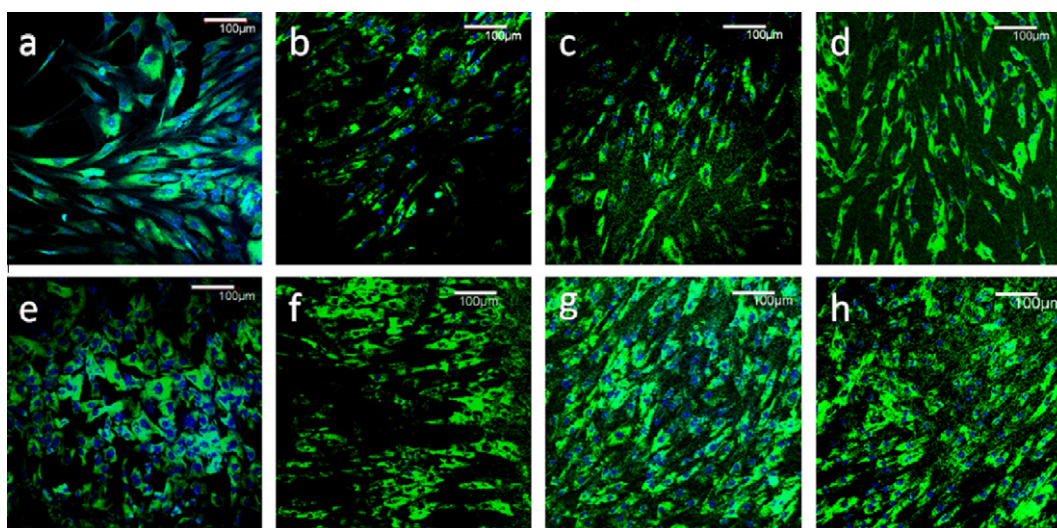


Fig. 4. Confocal microscopy image of hMSC on (a, e) TCP, (b, f) electrospun PLLACL, (c, g) PLLACL–collagen 3:1 and (d, h) PLLACL–collagen 1:1. (a–d) Cells were stained after 7 days culture; (e–h) cells were stained after 14 days culture. Scale bar 100 μm .

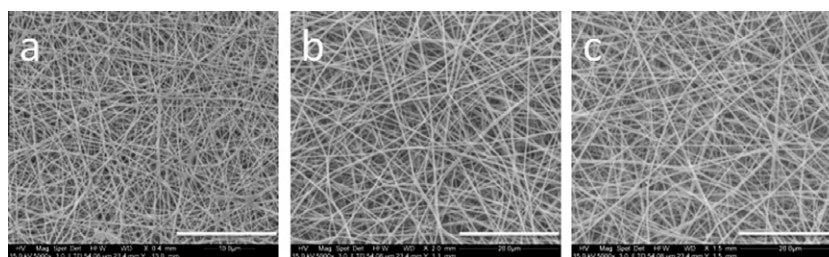


Fig. 5. SEM images of nanofibers loaded with DEX and BMP2: (a) blended nanofibers; (b) DEX in the shell/BMP2 in the core; (c) both DEX and BMP2 in the core. Scale bar 20 μm .

pronounced on blended collagen–PLLACL nanofibers compared with the PLLACL nanofibers [17].

3.3. The morphology of DEX and BMP2 loaded nanofibers

The morphology of the DEX and BMP2 loaded nanofibers is shown in Fig. 5. The smooth surfaces of the nanofibers indicated that both DEX and BMP2 were successfully incorporated into the resultant core–shell fibers. Moreover, it seems that the fibers electrospun from different solutions did not show appreciable differences in morphology.

FITC–BSA was used for the study of the core–shell structure of the nanofibers [8]. Fig. 6a indicates the distribution of proteins in the core–shell fibers. The fibers emitted fluorescent light, suggesting the presence of FITC–BSA. The results confirm that the proteins were distributed in the core of the PLLACL–collagen fibers. Fig. 6b shows TEM micrographs of the core–shell structure of PLLACL–collagen nanofibers loaded with DEX in the shell and BSA + BMP2 in the core of the nanofibers.

3.4. In vitro growth factor release

The controlled release of BSA and DEX from composite fibrous mats were studied and their release profiles from electrospun nanofibrous mats at a temperature of 37 $^{\circ}\text{C}$ and pH 7.4 are shown in Figs. 7–9. The experiments were performed in triplicate and the error bars indicate the standard deviation. The release profile of the drugs was studied at three stages: an initial burst release (stage I), followed by decreasing release (stage II), followed by constant release

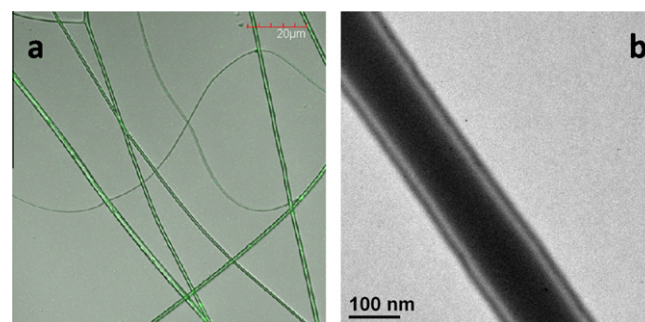


Fig. 6. (a) Fluorescence microscopic image of PLLACL–collagen core–shell fibers, with FITC–BSA and BMP2 in the core and DEX in the shell. An overlay of fluorescence and optical images. Scale bar 20 μm . (b) TEM micrographs of the core–shell structure.

(stage III). There was an initial burst release of DEX and BSA (within 6 h) from the blended nanofibrous mat (mat A) during stage I, shown in Fig. 7, with 43.8% and 48.5%, respectively, of the agents released. Between 6 and 72 h (stage II) the release curves exhibited a decreasing rate of release. The amounts of DEX and BSA released reached 64.1% and 70.3%, respectively. After 72 h the amount of drug and protein released from the blended nanofibers achieved a constant rate, reaching 70.8% and 81.1%, respectively.

BSA and DEX release from the coaxial electrospun composite membranes (mat B) were also examined, and are shown in Fig. 8. Fig. 8 shows the release profiles of the drug and protein from nanofibrous mat B in the same environment.

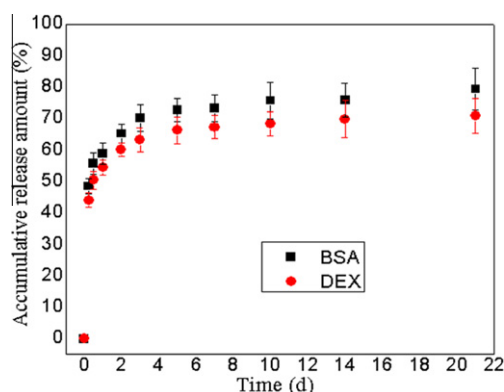


Fig. 7. Release profiles of BSA and DEX from blended fibrous mat A (blended).

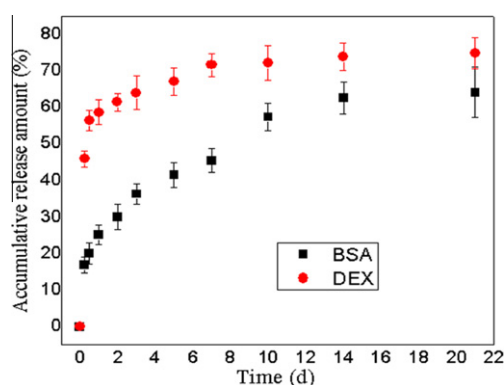


Fig. 8. Release profiles of BSA and DEX from coaxial fibrous mat B (DEX in the shell/ BMP2 in the core).

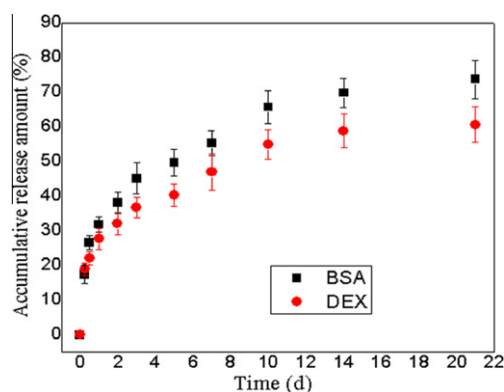
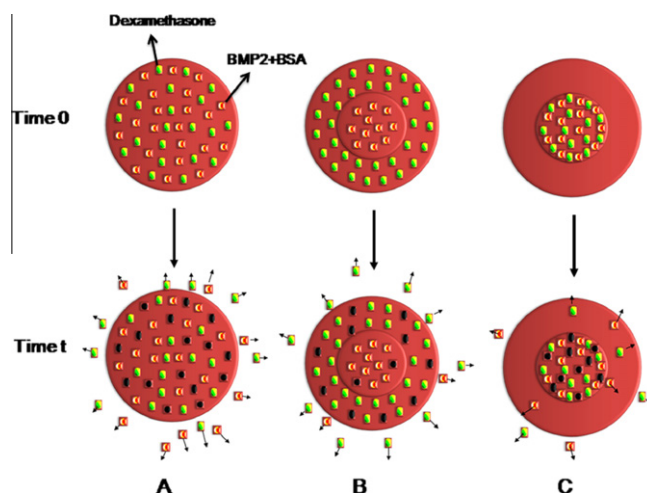


Fig. 9. Release profiles of BSA and DEX from coaxial fibrous mat C (both DEX and BMP2 in the core).

The release profile for BSA showed a less dramatic initial release which was initially slow and steadily increased. The rate of release of BSA accelerated temporarily after 168 h before subsequently becoming constant. The release profile for DEX showed dramatic initial release before it subsequently became constant.

At the end of the release study the percentage of DEX released was about 74.9%, while the percentage of BSA release was only 64.2%, because BSA was incorporated in the core, while DEX was located in the shell layer of the nanofibers.

Fig. 9 shows the cumulative release profiles of BSA and DEX from coaxial electrospun composite fibrous mat C. It shows a similar profile of BSA and DEX release from mat C. Both BSA and DEX showed a slight burst release within the first 6 h (BSA 17.4%, DEX



Scheme 2. A schematic representation of the release of DEX and BMP2 from electrospun nanofibers A, B and C.

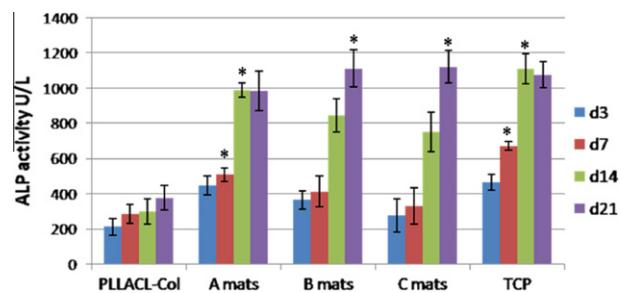


Fig. 10. Alkaline phosphatase (ALP) activity on PLLACL–collagen nanofibrous mats loaded with BMP2 and DEX after 3, 7, 14 and 21 days culture. *A significant difference between the different scaffolds was noted as ($P < 0.05$).

18.9%) (stage I). Subsequently the release of DEX and BSA became stable and sustained. After 168 h the release rate of DEX and BSA increased (stage II), followed by constant release (stage III). The amount of BSA and DEX released reached 73.7% and 60.5%, respectively, after 504 h. Scheme 2 also illustrates how DEX and BMP2 were encapsulated and released from the three different types of nanofibers.

The mechanism of release of the two growth factors is affected by two main considerations, the first being diffusion and the second being degradation of the polymer. From the release profiles the mechanism of release of DEX and BSA seems to be via diffusion and an erosion-coupled mechanism. The drugs were initially released from the fibrous mats by diffusion through the pores of the fibers. This changed to a combined diffusion/erosion-coupled mechanism later on [35]. The experimental results also demonstrate that release of the drug and/or protein can be influenced and varied by altering the method of electrospinning, namely blending or coaxial electrospinning. Furthermore, the diffusion rate is affected by the ratio of the thickness of the shell to that of the core and thus adjusting this ratio can affect the drug release rate. When the drug and/or protein is encapsulated in the core of the nanofibers the release profile presents a slow and steadily increasing profile, however, when the drug and/or protein is in the shell of the nanofibers there is an initial burst release at the beginning of the release process.

3.5. Bioactivity of DEX and BMP2 released from the nanofibers

ALP activity was measured as a marker of osteogenic differentiation, which plays a major role in the formation of mineral deposits

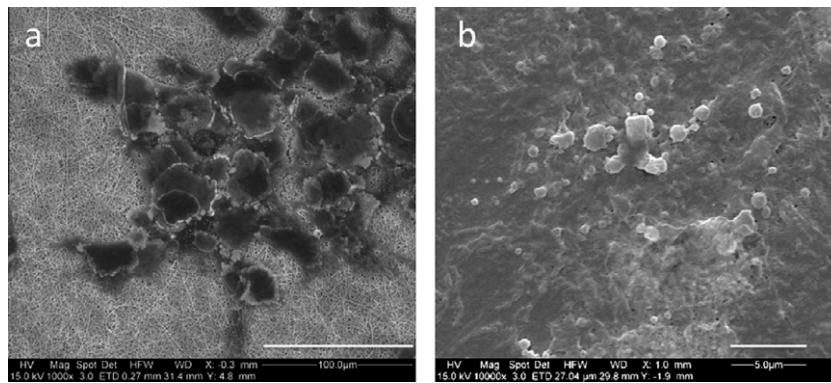


Fig. 11. (a) SEM images of hMSC cultured on coaxial nanofibrous mat B (DEX in shell and BMP2 in core) for 14 days. Scale bar 100 μm . (b) Mineralization of nodules produced by hMSC shown at higher magnification. Scale bar 5.0 μm .

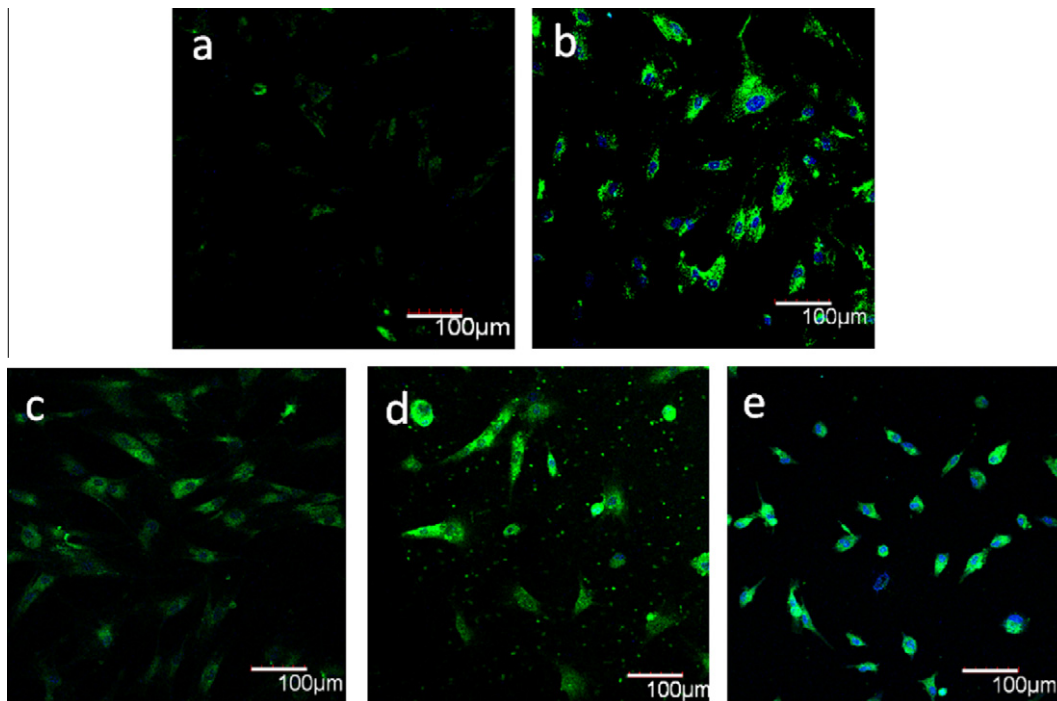


Fig. 12. Osteocalcin staining of hMSC on substrates on day 21: (a) PLLACL–collagen nanofibers; (b) TCP; (c) blended nanofibers; (d) DEX in the shell/BMP2 in the core; (e) both DEX and BMP2 in the core. Scale bar 100 μm .

in the matrix during bone tissue engineering. It can be observed in Fig. 10 that ALP activity increased with increasing culture time for all the test groups. It can also be seen that this increase is more pronounced for nanofibers loaded with BMP2 and DEX (mats A, B and C) compared with the PLLACL–collagen nanofibers.

It can also be seen that ALP activity of cells on mat A for the first three culture times of 3, 7 and 14 days were significantly higher than those on mats B and C. The reason for the higher ALP activity is the burst release of BMP2 from mat A and thus a higher concentration of growth factor in the medium for mat A than for mats B and C.

However, by day 21 the ALP activity of cells on mat A dropped and was lower than that of cells on mats B and C, because the rate of release of BMP2 from mat A became constant in the later stages while the rate of release from mats B and C continued to increase, at a decreasing rate, at later time points. As a result, the concentration of growth factor is higher in mats B and C during longer periods of cell culture.

The TCP control gave a better result than the mats containing BMP2 and DEX. BMP2 and DEX were added to the medium of the TCP controls. However, BMP2 and DEX released from the mats may have been removed when the medium was changed. As a result, the concentrations of BMP2 and DEX in the medium of the mats containing BMP2 and DEX was lower than those in the medium of the TCP controls.

The morphology of hMSC cultured on nanofibers loaded with BMP2 and DEX was observed by SEM and is shown in Fig. 11. The image at higher magnification clearly shows the deposition of minerals on day 14, with differentiation of hMSC into osteoblasts. In mat B DEX was loaded in the shell and BMP2 was located in the core of the nanofiber. Thus there was an initial dramatic burst of DEX release from mat B which subsequently became constant, however, the BMP2 in mat B was released in a stable and steadily increasing manner. This shows that mat B contained enough of the growth factors throughout the release period to induce hMSC differentiation to osteoblasts.

The osteogenic phenotype of hMSC seeded on nanofibers with and without DEX and BMP2 was observed by immunocytochemical staining for osteocalcin (a specific marker for the late stage of osteogenic differentiation). LSCM images show osteocalcin expression by hMSC on the growth factor loaded nanofibers after 21 days in culture. In Fig. 12 it can be observed that there was less protein expression by cells on PLLACL–collagen nanofibers than on fibers loaded with growth factors. Protein expression was significantly higher in cells on nanofibers loaded with growth factors, and it was also observed to be higher in hMSC cultured on the control TCP in standard osteogenic differentiation medium. Osteocalcin expression was weaker on blended nanofibers than on coaxial nanofibers. A possible reason is that most of the growth factors were released in an initial burst and were removed when the medium was changed over the first 7 days of culture and thus were unavailable to support osteogenic differentiation.

Drug carrier systems are designed to maximize the therapeutic activity while at the same time minimizing any toxic side-effects of the drug(s) [36]. In our experiments our drug loaded nanofiber device was designed to operate as an ECM for hMSC growth and supply differentiation factors to adherent cells. It has been shown that osteogenic differentiation is sensitive to the dose and duration of exposure to DEX and BMP2 [27,37]. As such, it would be ideal to supply the appropriate amount of drug needed to promote proliferation and differentiation towards osteogenic cells. To achieve that we have developed a drug delivery system to release different drugs at different stages.

Based on our experiments, BMP2 and DEX with constant bioactivity were released and their effects on cell differentiation were significant. This can be seen in the increase in ALP expression and the deposition of minerals produced by differentiated cells cultured on nanofibers loaded with the drugs. Immunocytochemical staining of the protein osteocalcin confirmed that osteoblastic differentiation from hMSC had occurred. These results show the need for further research and exploration so as to create drug carrier devices that hold large amounts of drug and yet at the same time are able to release exact amounts of drug at specific time intervals, thus reducing the need for constant replenishment of the drug, without the harmful effects of a drug overdose. This is particularly useful in surgical applications that require drug delivery at specific time intervals but for which replenishment would mean further surgery that would put the patient at a higher risk of medical complications.

4. Conclusion

PLLACL–collagen (3:1) nanofibrous mats have great potential for bone tissue engineering because of their hydrophilicity and ability to support the proliferation of hMSC. Coaxial electrospinning can also be used to load the PLLACL–collagen nanofibers with two different growth factors, loading these growth factors in different layers of the PLLACL–collagen nanofibers. When DEX is loaded into the shell layer it shows a sharp initial burst release while BSA–BMP2 loaded into the core of the fiber results in slow and steady long-term release from coaxial electrospun fibers. Such a release profile where there are sufficient amounts of growth factors throughout the entire release period can help induce hMSC differentiation into osteogenic cells. Increased ALP activity, mineralization, and osteoblast marker expression have been assessed on nanofibers loaded with the two growth factors. These experimental results show that this two growth factor system of coaxial electrospun nanofibers has great potential for applications in bone tissue regeneration and could serve as a platform for other researchers to carry out further studies.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figures 2, 4, 6–10, 12, schemes 1 and 2 are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi: 10.1016/j.actbio.2011.11.002.

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