Microsphere-based drug releasing scaffolds for inducing osteogenesis of human mesenchymal stem cells in vitro

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ABSTRACT

In this study, in vitro osteogenesis was successfully achieved in human mesenchymal stem cells (hMSCs) by controlled release of the osteogenesis-inducing drugs dexamethasone, ascorbic acid (AA) and β-glycerophosphate (GP) from poly(lactic-co-glycolic acid) (PLGA) sintered microsphere scaffolds (SMS). We investigated the osteogenesis of human MSCs (hMSCs) on dexamethasone laden PLGA-SMS (PLGA-Dex-SMS), and dexamethasone, AA and GP laden PLGA-SMS (PLGA-Com-SMS). hMSCs cultured on the microsphere systems, which act as drug release vehicles and also promote cell growth/tissue formation—displayed a strong osteogenic commitment locally. The osteogenic commitment of hMSCs on the scaffolds were verified by alkaline phosphatase (ALP) activity assay, calcium secretion assay, real-time PCR and immunohistochemistry analysis. The results indicated hMSCs cultured on PLGA-Com-SMS exhibited superior osteogenic differentiation owing to significantly high phenotypic expression of typical osteogenic genes—osteocalcin (OC), type I collagen, alkaline phosphatase (ALP), and Runx-2/Cbfa-1, and protein secretion of bone-relevant markers such as osteoclast and type I collagen when compared with PLGA-Dex-SMS. In conclusion, by promoting osteogenic development of hMSCs in vitro, this newly designed controlled release system opens a new door to bone reparation and regeneration.

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1. Introduction

Self-repair of bone tissue depends on the defect size and host source of osteoprogenitors (Elaine et al., 2008; Meyer and Wiesmann, 2006). Whereas minor injuries heal spontaneously, critical size defects will not completely repair by this self-regeneration process (Elaine et al., 2008). Moreover, the defects caused by trauma, tumors and infections will compromise the host source of osteoprogenitors. Thus dependence on only the local osteoprogenitors for bone repair is not practical (Meyer and Wiesmann, 2006). Therefore, bone replacement procedures using autograft, allograft tissue or artificial materials have been proposed (Steffens et al., in press). However, limited source and immunological rejection restrict the administration of autograft and allograft

tissue replacement (Steffens et al., in press). Tissue engineering strategies can overcome the drawbacks in the utilization of autografts and allografts. Cell, scaffold and growth factor are the three important factors for tissue engineering approach (Biondi et al., 2008).

Mesenchymal stem cells (MSCs), which have the capacity to self-renew and differentiate into myocytes, osteoblasts, chondrocytes and adipocytes have drawn more and more attention to their application in bone repair therapy clinically (Uccelli et al., 2008). Directing MSC differentiation in vitro or in vivo is one of the critical challenges that need to be overcome (Hwang et al., 2008). Growth factors such as bone morphogenetic protein-2 (BMP-2) are the most widely used molecules for MSC osteogenesis and promoting new tissue formation (Allori et al., 2008). However, high administration dosage, short half-lives and ease of deactivation are the key drawbacks in the clinical applications of growth factors in bone repair (Service, 2000). Some chemical reagents such as dexamethasone (Nuttelman et al., 2006a), alendronate (AL) (von Konch et al., 2005) and Vitamin D3 (D'ippolito et al., 2002) are also utilized in MSCs osteogenic commitment, because they possess higher chemical and physical stability and also low cost.

Charles et al. developed a dexamethasone-functionalized gel for osteogenic differentiation (Nuttelman et al., 2006b). In this study, an easier and effective method was explored for osteogenic dif-

ferentiation of MSCs locally. Dexamethasone, AA and β-GP, which are the main efficacious components in traditional osteogenic media, were encapsulated into controlled release systems for MSC osteogenesis. Dexamethasone is a glucocorticoid, which is used extensively *in vitro* as an osteogenic factor, and *in vivo* for the treatment of inflammatory and autoimmune diseases. GP, which functions as a source of inorganic phosphate, promotes osteoblast differentiation and mineralization in primary culture of MSCs (Akavia et al., 2006; Bear et al., 2008; Anurag et al., 2007). AA is one of the essential supplements for the differentiation of osteoblast-like cells, which can stimulate collagen synthesis and ALP activity (Anurag et al., 2007; Takamizawa et al., 2004).

In order to achieve efficient osteogenesis of MSCs by treatment with osteogenesis-inducing drugs, a superior system for optimal drug delivery that also provides a suitable three-dimensional structure for cell attachment, proliferation and differentiation is in imperative demand (Lee and Shin, 2007). Sintered microsphere technique which sinters polymer microspheres by heat or solvents and constructs them together into a scaffold is a beneficial method for fabricating drug releasing scaffolds (Shi et al., 2009a; Borden et al., 2002). Besides retaining the favorable drug/protein encapsulation and controlled release capacities of the polymer microspheres which are extensively used as drug vehicles, sintered microsphere scaffold also possesses porous structures and good mechanical properties which are necessary characteristics of bone tissue engineering scaffolds (Wang et al., 2009; Shi et al., in press). Poly(lactic-co-glycolic acid) microsphere was adopted for scaffold fabrication in this study because of its controlled degradability and acknowledged drug/protein controlled release properties (Shi et al., 2009b; Kofron et al., 2009), and previous studies indicated PLGA sintered microspherical scaffold supported bone formation *in vivo* (Shi et al., 2009c).

In this study, osteogenesis-inducing drugs (dexamethasone, AA and GP) were encapsulated into PLGA sintered microsphere scaffolds (PLGA-SMS) and the osteogenesis of human MSCs (hMSCs) on dexamethasone laden PLGA-SMS (PLGA-Dex-SMS) and dexamethasone, AA and GP laden PLGA-SMS (PLGA-Com-SMS) was investigated.

2. Materials and methods

2.1. Materials

PLGA (lactic/glycolic 1:1; Mw 31,000 Da; inherent viscosity 0.30 dL/g in chloroform at 30 °C) was purchased from Daigang Biomaterials Inc. (Jinan, China). Poly(vinyl alcohol) (PVA), β-glycerophosphate, ascorbic acid and dexamethasone were obtained from Sigma–Aldrich (Singapore). All cell-culture-related reagents were purchased from Gibco (Invitrogen, Singapore).

2.2. Synthesis methods

2.2.1. PLGA-dexamethasone microspheres

PLGA microspheres encapsulating dexamethasone (10 mg) were prepared by single emulsion-solvent evaporation technique using our previously published method (Shi et al., 2009c). Briefly, PLGA (5 g) was dissolved in 25 ml dexamethasone–methylene chloride solution with 10 mg dexamethasone. The resulting oil phase (O) was added dropwise to 1000 ml 1% PVA solution (W), and then stirred at 200 rpm for 6 h to allow methylene chloride to evaporate completely. PLGA microspheres were collected and washed three times with deionized water, and then dried at room temperature for 24 h.

2.2.2. PLGA-Com microspheres

PLGA microspheres encapsulating dexamethasone-(β-GP)-AA were prepared by double emulsion-solvent evaporation technique using our previously published method (Shi et al., 2009c). Briefly, β-GP (4 g) and AA (0.2 g) were dissolved in 7 ml deionized water as the first water phase (W_1). PLGA (5 g) was dissolved in 25 ml dexamethasone–methylene chloride solution with 10 mg dexamethasone as oil phase (O). The W_1 solution was then emulsified in oil phase to form the primary W_1/O emulsion via homogenization at 5000 rpm for 30 s. The resulting emulsion was added dropwise to 1000 ml 1% PVA solution (W_2), and then stirred at 200 rpm for 4 h to allow methylene chloride to evaporate completely. The PLGA-Com microspheres were collected and washed three times with deionized water, and then dried at room temperature for 24 h.

2.2.3. PLGA-Dex-SMS and PLGA-Com-SMS

PLGA-Dex-SMS and PLGA-Com-SMS were fabricated via microsphere sintered technique. Briefly, the microspheres (100 mg) were stacked in an open mold (diameter = 10 mm, height = 5 mm). 0.6 ml acetone/ethanol solvent (20%, v/v) were added into the mold and placed for 30 s which made the microspheres fuse into a scaffold (Brown et al., 2008). The scaffold was then washed with deionized water and separated from the mold. PLGA-SMS without drug laden was used as control in this study.

2.3. Morphology

Morphology of scaffolds (cylindrical scaffolds, diameter = 10 mm, height = 5 mm) was conducted using scanning electron microscopy (SEM, 30XLFEG, Philips, The Netherlands). The microspheres were immobilized on a copper stub and coated with gold by a Ion Sputte (HCP-2, HITACHI, Japan), and the voltage for morphologic analysis is 5.0 kV.

2.4. Porosity determination of scaffolds

Ethanol was used as the liquid phase and kept at 25 °C. A bottle filled with ethanol was weighed (W_1). Then a sintered microspherical scaffold sample (cylindrical scaffolds, diameter = 10 mm, height = 5 mm) weighing W_S was immersed into the bottle and weighed (W_2). ρ is the density of ethanol at 25 °C. The size of the cylindrical scaffold including radius (R) and height (H) was measured. The porosity (P) was calculated using the equation as follows:

$$P = 1 - \frac{((W_1 - W_2 + Ws)/\rho)}{(\pi \times R^2) \times H} \tag{1}$$

2.5. Determination of drug encapsulation efficiency

An accurately weighed amount (20 mg) of sintered microspherical scaffold was dissolved in 1 ml methylene chloride, and additional 4 ml methylene chloride (for the determination of dexamethasone) or 5 ml 0.9% NaCl solution (for the determination of β-GP and AA) was added subsequently. This solution was stirred at 37 °C for 2 h. After centrifugation, dexamethasone in the oil phase and, β-GP and AA in the aqueous supernatant were analyzed using different methods, respectively. Briefly, dexamethasone was analyzed using high performance liquid chromatography (HPLC, Shimadzu, Japan) with a 150 mm × 6.0 mm column. Acetonitrile, phosphate buffer solution and methanol were utilized as mobile phase, and the detector response was measured at 254 nm (Lqbal et al., 2006). β-GP and AA were analyzed using a molybdenum blue method by UV spectrophotometer (Multiskan® Spectrum, Thermo, Finland) according to a previously published method (Shen and

Dyroff, 1962). Encapsulation efficiency (EE) was expressed by equation as follows:

$$EE = \left(\frac{\text{actual drug loading}}{\text{theoretical drug loading}} \right) \times 100 \qquad (2)$$

2.6. In vitro release test

Dexamethasone, β-GP and AA release were determined by suspending 100 mg sintered microsphere scaffolds in 50 ml PBS buffer (for the determination of dexamethasone) and in 50 ml 0.9% NaCl solution (for the determination of β-GP and AA), respectively. Five hundred microlitres of the sample media were collected periodically with equal amount of PBS or NaCl solution makeup. Drug amount at every time point was measured as the method described in Section 2.5.

2.7. Cell culture

Human bone marrow mesenchymal stem cells (Bone marrow hMSCs) purchased from Lonza Walkersville Inc. (Walkersville, MD, USA) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Passage 5 hMSCs were used in this study.

2.8. Cell seeding

PLGA-Dex-SMS (weight: 100 mg, with 200 μg dexamethasone/scaffold) and PLGA-Com-SMS (weight: 100 mg, with 200 μg dexamethasone/scaffold) were placed in 24-well TCPS (tissue culture polystyrene) plates, and the bottoms of which were pre-coated with sterile agarose gels. Fifty-microlitre cell suspension (4×10^6 cells/ml) were seeded on the scaffolds and incubated in a humid atmosphere under 37 °C and 5% CO_2 in DMEM with 10% FBS. After 3 days, the medium (1 ml) was changed to remove non-adherent cells, and the medium was subsequently changed every 3 days. hMSCs cultured on PLGA-SMS not encapsulating any drugs were used as a control.

2.9. Cell proliferation

Cell number was determined with a DNA assay using Sigma quantification kit (Sigma–Aldrich, Singapore) following the manufacturer's instruction. Via the measured DNA quantities, the exact numbers of the committed cells could be counted using a conversion rate of 6.6 pg DNA per cell. Cell proliferation on the scaffolds was detected on Day 7 and Day 21 using a "Live/Dead" assay (Molecular Probes, Invitrogen, Singapore). The specimens were observed under fluorescence microscope (Olympus, IX71, Japan).

2.10. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined using pNPP assay (p-nitrophenyl phosphate liquid substrate, Sigma Diagnostics). Briefly, the scaffolds with cells were pre-washed with PBS, and then the adherent MSCs were removed from the scaffolds and lysed in 0.5 ml PBS containing 0.1 M glycine, 1 mM $MgCl_2$ and 0.05% Triton X-100 for 10 min at 4 °C. The lysate was incubated with p-nitrophenyl phosphate (pNPP) solution at 37 °C for 30 min, and then subjected to a spectrophotometer (Multiskan® Spectrum, Thermo, Finland) on which the absorbance at 405 nm was measured and recorded to indicate ALP concentration. ALP activity was calculated from a formula offered by manufacturer

after normalizing cell number. Drug-free PLGA scaffold was used as control.

2.11. Calcium secretion assays

A semi-quantificational Alizarin red-based assay of mineralization was performed after 7, 14 and 21 days of culture according to Ref. (Gregory et al., 2004). Briefly, the scaffolds were washed twice with excess dH_2O prior to the addition of 1 mL of 40 mM alizarin red (pH 4.1) per well. The plates were incubated at room temperature for 20 min with gentle shaking. Stained mineralized layer was extracted by 10% (v/v) acetic acid, and Aliquots (150 μL) of the extracted solution were read at 405 nm using a spectrophotometer (Multiskan® Spectrum, Thermo, Finland).

2.12. Real-time quantitative reverse transcription-polymerase chain reaction (real-time RT-PCR

Real-time RT-PCR was performed as follows: total RNA was isolated from cells which have underwent osteogeneic differentiation for 14 days and 28 days following TRIzol protocol and subjected to reverse transcription with SuperScript™ First-Strand Synthesis System (Promega, USA). The complementary DNA (cDNA) yield was then subjected to PCR to examine the gene expression of osteocalcin (OC), type I collagen, alkaline phosphatase (ALP), Runx-2/Cbfa-1, and β-actin. The adopted primer sequences are list as follows: β-actin (5′-CCTGGCACCCAGCACAAT-3′ and 5′-GGGCCG-GACTCGTCATACT-3′), OC (5′-AGCAAAGGTGCAGCCTTTGT-3′ and 5′-GCGCCTGGGTCTCTTCACT-3′), ALP (5′-GGGAACGAGGTCA-CCTCCAT-3′ and 5′-TGGTCACAATGCCCACAGAT-3′), type I collagen (5′-CCTGCGTGTACCCCA-CTCA-3′ and 5′-ACCAGACAT-GCCTCTTGTCCTT-3′) and Runx-2/Cbfa-1 (5′-TGATG-ACACTGCCACCTCTGA-3′ and 5′-GCACCTGCCTGGCTCTTCT-3′). Quantitative polymerase chain reaction (qPCR) was conducted using SYBR green assay (iQ supremix, Bio-rad). The gene expressions were quantified by calculating $2^{-\Delta C_T}$ values, where C_T represents the cycle number when an arbitrarily placed threshold was reached, and $\Delta C_T = (C_{T, \text{target gene}} - C_{T, \beta\text{-actin}})$.

2.13. Immunofluorescence staining

Immunofluorescence staining was conducted as follows: after 21 days culture, cell laden microspheres were fixed in 4% paraformaldehyde solution for 30 min. Thereafter the fixed specimens were incubated in 10% goat blocking serum for 1 h to suppress non-specific binding of IgG. The specimens were then incubated in the collagen I primary antibody (2 ng/mL, mouse monoclonal IgG, Santa Cruz Biotechnology) or osteocalcin primary antibody (2 ng/mL, rabbit polyclonal IgG) at 37 °C for 1 h and anti-IgG (5 ng/mL, Invitrogen Alexa Fluor, 546) at room temperature for 1 h followed by three washes in PBS. The specimens were then observed under fluorescent microscope (Olympus, IX71, Japan).

2.14. Statistical analysis

Experiments were repeated six times and results were expressed as means ± standard deviations. Statistical significance was calculated using one-way analysis of variance (one-way ANOVA). Comparison between the two means was performed using Tukey test and data was determined as statistically significant for $p < 0.05$.
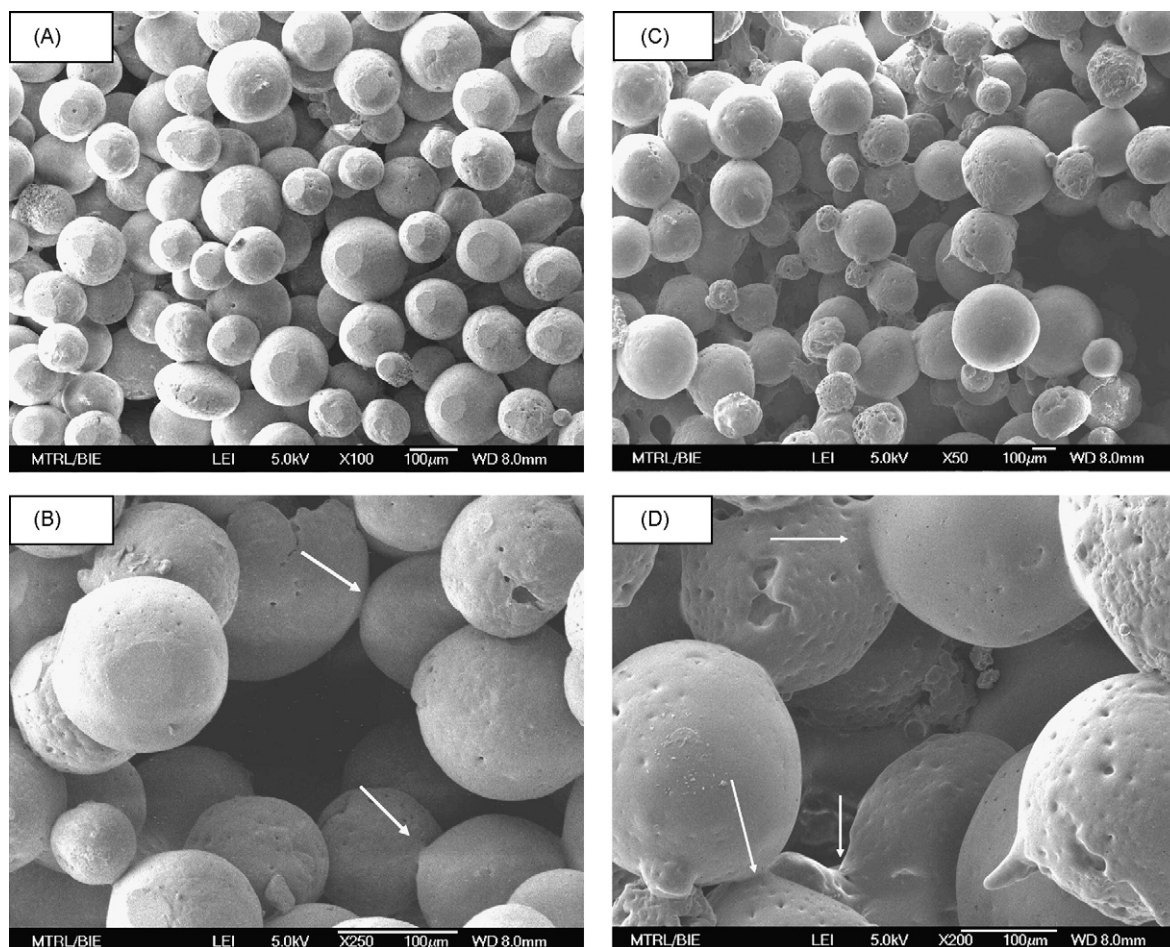
Fig. 1. The morphology of PLGA-Dex-SMS (A and B) and PLGA-Com-SMS (C and D).

3. Results

3.1. Morphology of microsphere-based scaffolds

PLGA-Dex and PLGA-Com microspheres were fabricated via single emulsion and double emulsion techniques, respectively. PLGA-Com-SMS and PLGA-Dex-SMS were produced by solvent sintering technology. Their respective morphologies are shown in Fig. 1. After being sintered with 20% acetone/ethanol solvent, composite microspheres still maintain their spherical shape, and remarkable bonding regions could be found among microspheres in both the scaffolds. The visible large pores (more than 100 μm) which will facilitate cell ingrowth and vascular invasion could be observed in the scaffolds. The porosities of PLGA-Dex-SMS and PLGA-Com-SMS were (33.9 ± 10.0)% and (32.5 ± 6.1)%, respectively.

3.2. Drug encapsulation efficiency and delivery

In this study, the dexamethasone encapsulation efficiency of PLGA-Dex-SMS and PLGA-Com-SMS was (89.3 ± 5.2)% and (82.5 ± 1.9)%, respectively. The β-GP and AA encapsulation efficiency of PLGA-Com-SMS was (27.2 ± 2.3)% and (11.8 ± 5.6)%, respectively.

The dexamethasone release profiles of PLGA-Dex-SMS and PLGA-Com-SMS are shown in Fig. 2(A). The release profiles exhibit an analogously exponential tendency, and the burst release of dexamethasone from PLGA-Dex-SMS and PLGA-Com-SMS for the first 5 days is (31.9 ± 3.1)% and (43.6 ± 4.6)% of total dexamethasone loading amount, respectively. It can also be observed that the dexamethasone release curves of PLGA-Dex-SMS and PLGA-Com-SMS reach equilibrium concentration of around 90% and 100% on Day 36, respectively.

The profiles of β-GP and AA release from PLGA-Com-SMS over 36 days are shown in Fig. 2(B). The curve of AA exhibits a significant bursting initiation. According to the trend, (89.2 ± 3.9)% of total AA loading amount was released from PLGA-Com-SMS during the first 5 days. In contrast, the profile of β-GP release indicates a minimal initial burst release [(45.2 ± 2.7)% of total β-GP loading amount]. Almost all the laden AA and β-GP molecules in PLGA-Com-SMS were released into aqueous solution on Day 20 and Day 24, respectively.

3.3. Cell proliferation

Cell morphology and distribution on the scaffolds (Fig. 3) was recorded by fluorescence microscopy, and cell number on each scaffold was also measured. As seen from the micrograph, cell attachment and proliferation mainly occurred around the microspherical brim on Day 7, and after 21 days of culture, a significantly larger cell number was found on the control sample [$(25.7 \pm 1.7) \times 10^4$/scaffold)] than that on PLGA-Dex-SMS [$(15.2 \pm 2.2) \times 10^4$/scaffold] and PLGA-Com-SMS [$(14.7 \pm 2.9) \times 10^4$/scaffold]. The cell number on PLGA-Dex-SMS was comparable to that on PLGA-Com-SMS.
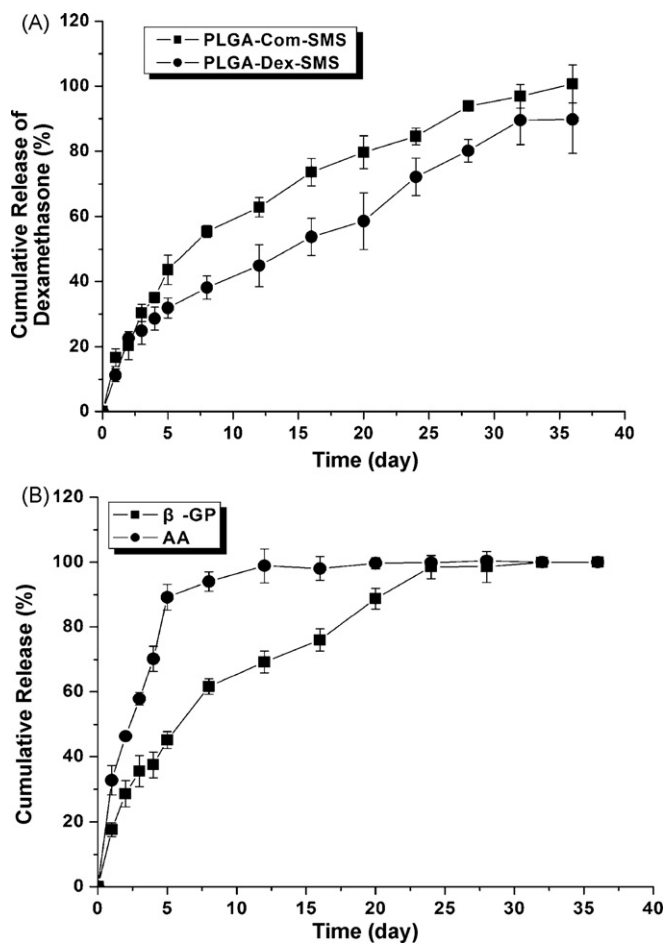
Fig. 2. Cumulative release of dexamethasone from PLGA-Dex-SMS and PLGA-Com-SMS (A), and AA and β-GP from PLGA-Com-SMS (B).

3.4. Osteogenesis

The secretion of ALP, a standard maker of osteogenic differentiation, was determined by pNPP assay (Fig. 4). Positive ALP production was detected at Day 7, followed by significant upregulation on Day 14 and slight downregulation on Day 21. hMSCs cultured on PLGA-Com-SMS exhibited significantly higher ALP activity than hMSCs on PLGA-Dex-SMS.

Calcium deposition by hMSCs was assessed by semi-quantificational alizarin red-based assays at Day 7, Day 14 and Day 21 (Fig. 5). After 14 days of culture, a significantly higher level of calcium secretion was observed from cells cultured on PLGA-Com-SMS. Calcium secretion by cells cultured on PLGA-Com-SMS was more than twice as that by cells on PLGA-Dex-SMS. Contrastively, there was no remarkable calcium secretion in hMSCs cultured on drug-free PLGA scaffold.

Gene expression of bone-relevant markers in hMSCs was determined by real-time RT-PCR at Day 14 and Day 28 (Fig. 6). Cells cultured on PLGA-Com-SMS had significantly higher expression of the well acknowledged early and late bone markers—Runx-2/Cbfa-1 and OC, and other bone-relevant markers—type I collagen and ALP, as shown in Fig. 6. The expression of Runx-2/Cbfa-1 in cells cultured on PLGA-Dex-SMS is comparable to that in cells cultured on PLGA-Com-SMS at Day 14.

Immunofluorescence staining of cellular aggregates (for type I collagen and OC) cultured on PLGA-Com-SMS, PLGA-Dex-SMS and PLGA-SMS is shown in Fig. 7. After 14 days of culture, type I col-
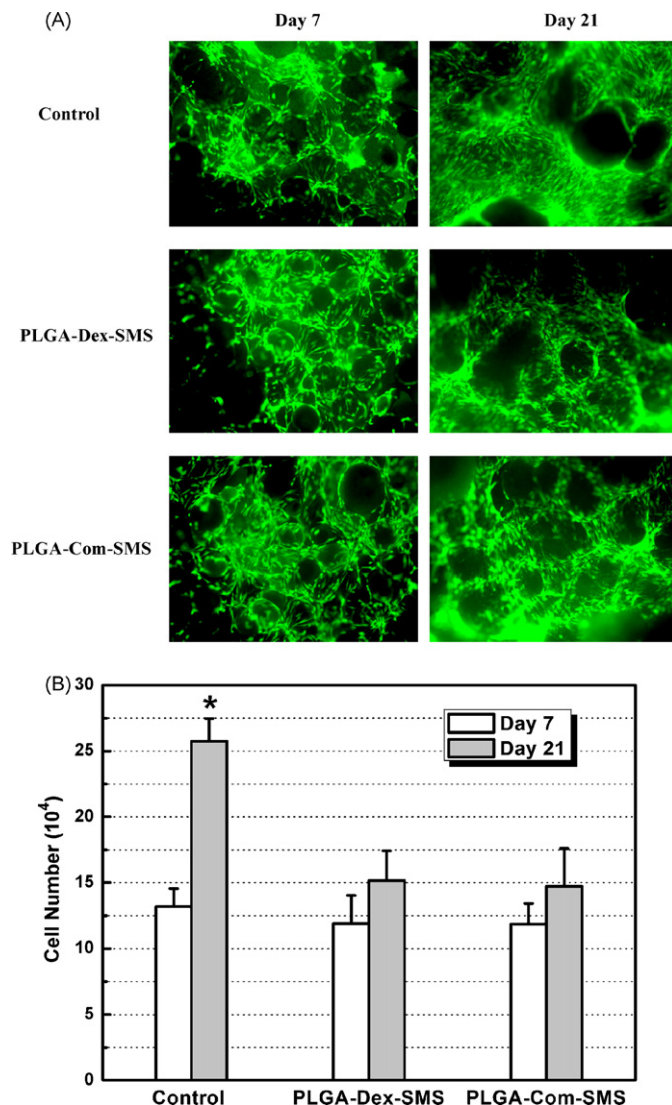

Fig. 3. Cell proliferation (A) and cell number (B) on PLGA-Dex-SMS and PLGA-Com-SMS after 7 and 21 days of culture. Asterisk (*) indicates statistical significance when compared with the PLGA-Dex-SMS and PLGA-Com-SMS ($p < 0.05$).
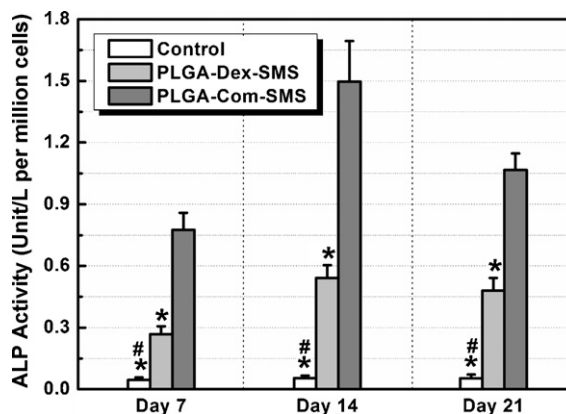

Fig. 4. ALP activity of hMSCs after 7 days, 14 days, and 21 days of culture. (#) and (*) indicate statistical significance when compared with the PLGA-Dex-SMS and PLGA-Com-SMS ($p < 0.05$), respectively.
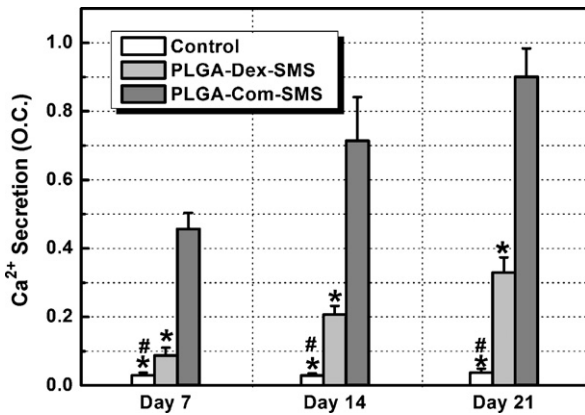
Fig. 5. Calcium secretion by hMSCs cultured on PLGA-Dex-SMS, PLGA-Com-SMS, and the control (PLGA-SMS) using semi-quantificational alizarin red-based assays. (#) and (*) indicate statistical significance when compared with PLGA-Dex-SMS and PLGA-Com-SMS ($p < 0.05$), respectively.

lagen and OC secreted from hMSCs cultured on PLGA-Com-SMS display grossly intensive fluorescence. However, there is weaker fluorescence staining of cells on PLGA-Dex-SMS, indicating weaker osteogenic capacity of hMSCs cultured on PLGA-Dex-SMS.

4. Discussions

Using specific growth factors to stimulate MSC differentiation and improve new functional tissue formation is a mainstream tissue engineering strategy for tissue repair and regeneration (Ma and Choi, 2001). However, these proteins possess short *in vivo* half-lives and also need high administration dosage (Service, 2000). In this study, we used a different approach for inducing MSC differentiation locally. Dexamethasone, β-GP and AA, which are the key components of osteogenic media, were introduced into drug releasing scaffolds for hMSC osteogenesis. In contrast with protein-based growth factors such as BMP-2, these chemicals tend to have high stability and longer active half-life in solution, which would prolong the interaction period between drug molecules and cells.

To achieve an ideally controlled release of dexamethasone–β-GP–AA during processing or application, the optional carriers must be able to ensure the retention of drugs, adequate drug release dosage and simultaneously avoid side effects. Considering the hydrophobicity of dexamethasone and hydrophilicity of β-GP and AA, PLGA microsphere-based scaffolds were used as ideal drug-encapsulating vehicles. Dexamethasone can hybridize with PLGA when PLGA is dissolved in dexamethasone–methylene chloride solution; while the hydrophilic drugs can be encapsulated into PLGA microspheres via typical double emulsion techniques. Dexamethasone and some other osteogenic factors laden PLGA particulate leaching scaffold (Kim et al., 2005) and chitosan-based particles (Oliveira et al., 2009) were also reported as drug and cell vehicles for MSCs' osteogenic differentiation. Compared with these scaffolds, as the essential ingredient of sintered microsphere scaffold, polymer microsphere still holds its advantage on drug encapsulation and release (Hosseinkhani et al., 2007). Besides acting as a drug carrier, PLGA microsphere scaffolds also offer a three-dimensional structure for cell proliferation and tissue ingrowth. These scaffolds qualify as bifunctional systems that can be used for both controlled release and tissue repair. As demonstrated in our previous study, the mechanical properties of the scaffolds are similar to that of human cancellous bone (Lee and Shin, 2007; Wang et al., 2009). Therefore, during bone recovery process, the scaffolds act as temporary substitution for defect bone tissue. Secondly, the porosity of the scaffolds is between 30 and
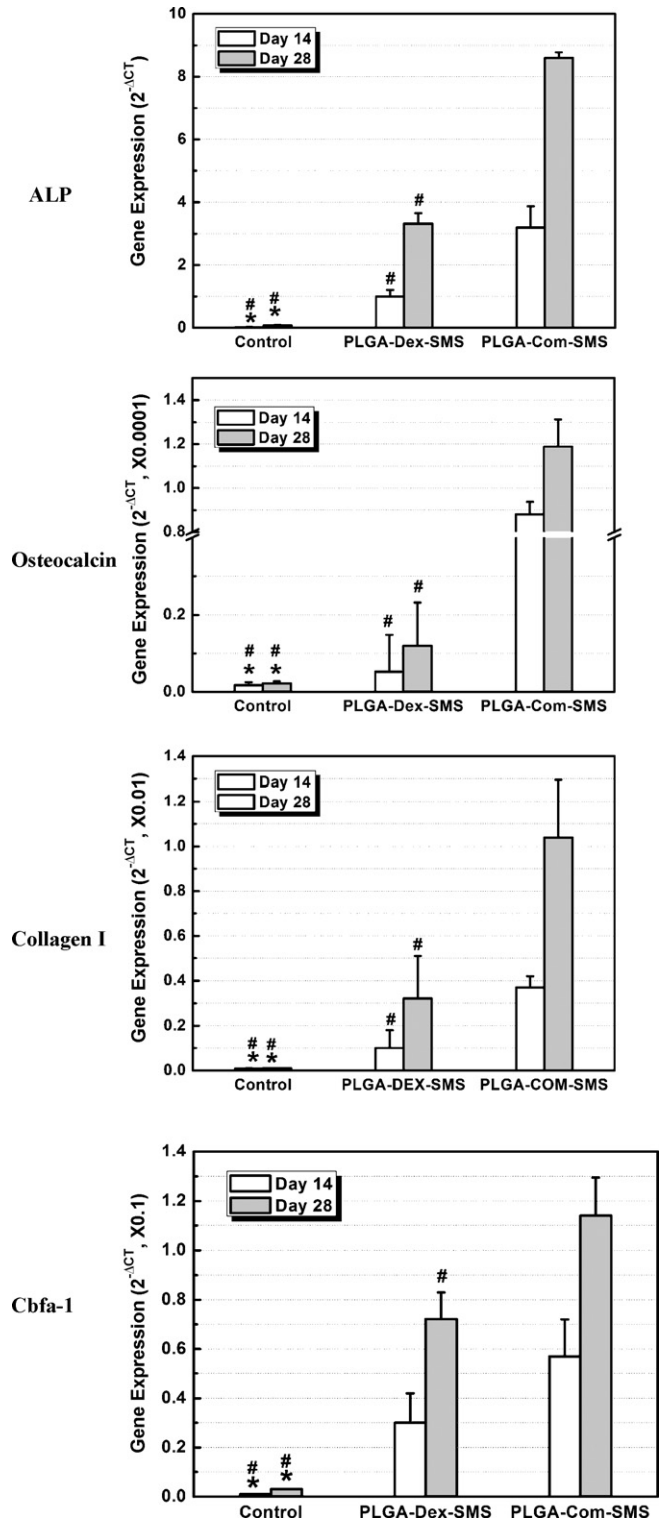

Fig. 6. Gene expression of osteocalcin, ALP, Runx-2/Cbfa-1 and collagen I using real-time PCR. (*) and (#) indicate statistical significance when compared with PLGA-Dex-SMS and PLGA-Com-SMS ($p < 0.05$), respectively.

50%, sintered microsphere scaffolds with similar structure have confirmed to be capable of facilitating cell ingrowth and proliferation (Jiang et al., 2006). Visible macropores (more than 100 μm in size) have been observed on the scaffolds (Fig. 1). These pores can provide interspaces for the formation of new blood vessels. Previous studies indicate that PLGA sintered microsphere scaffolds meet
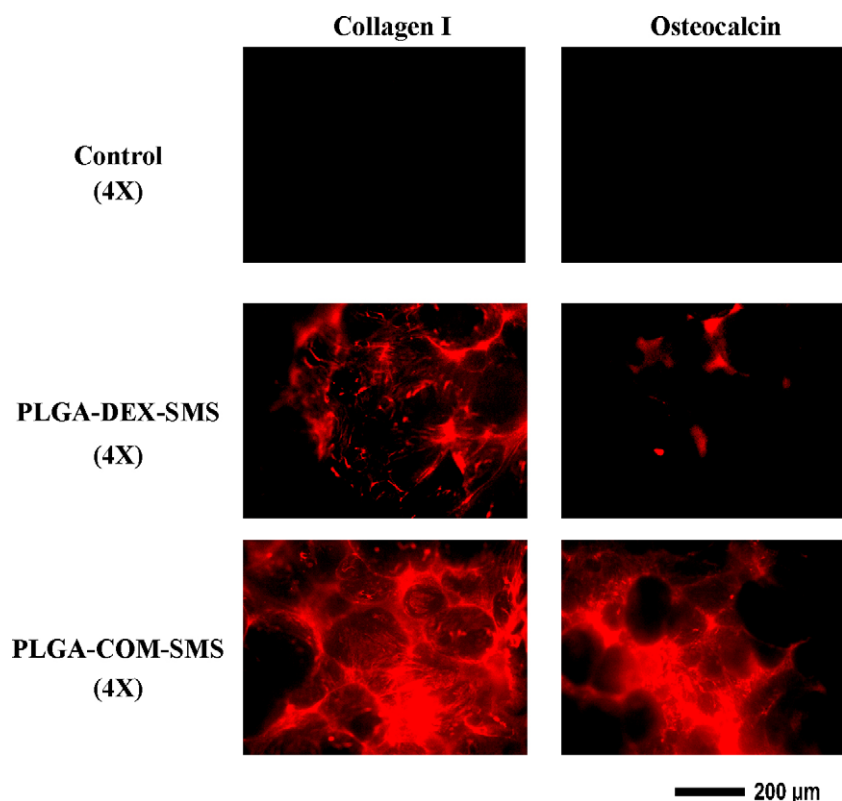
Fig. 7. Three-dimensional images for immunofluorescence staining of type I collagen and osteocalcin secreted by hMSCs cultured on PLGA-Dex-SMS and PLGA-Com-SMS for 14 days, respectively.

the demands for adipose-derived stromal cell osteogenesis and that endothelial cells can grow and maintain their characteristic phenotype for potential new blood vessel formation in the scaffolds (Shen et al., 2006; Jabbarzadeh et al., 2007).

As a synthetic glucocorticoid, dexamethasone exhibits both positive and negative effects on osteoblast differentiation (Philips et al., 2006). Dexamethasone is a stimulator of osteogenesis and contrastingly the long-term use of dexamethasone with high dosage *in vivo* causes severe osteoporosis (Jabbarzadeh et al., 2007; Philips et al., 2006; Walsh et al., 2001). Drug concentrations of over 4000 ng/ml of dexamethasone and 1600 μg/ml of AA cause significant cytotoxicity (ter Brugge and Jansen, 2002; Jaiswal et al., 1997; Kim et al., 2003). Therefore, the effective concentration of dexamethasone, β-GP and AA is a crucial factor for the MSCs osteogenic commitment. In this work, for the microsphere-based scaffolds, drug encapsulation and release rate can be predicted and controlled easily. The effective concentration of dexamethasone and AA for the osteogenic differentiation of MSCs was in the range of 40–400 ng/ml and 16–160 μg/ml (Jaiswal et al., 1997). The finalized PLGA-Dex-SMS and PLGA-Com-SMS have adequate encapsulated dexamethasone amount in 1 ml, which is $(178.6 \pm 10.4)\,\mu$g/scaffold and $(165.0 \pm 38.0)\,\mu$g/scaffold, respectively. The average dexamethasone release concentration in 1 ml culture medium or 50 ml PBS for PLGA-Dex-SMS and PLGA-Com-SMS is about 102.1 ng/ml/day and 94.2 ng/ml/day. β-GP and AA release concentration for PLGA-Com-SMS is 622.4 μg/ml/day and 13.5 μg/ml/day.

Owen et al. (Owen et al., 1990) reported that MSCs show lower proliferation and promotive osteogenic differentiation after treatment by cell culture medium with low dexamethasone concentration. Our findings in general agree with the study of Owen et al. As shown in Fig. 3, the cell number on dexamethasone-free

scaffold is overwhelmingly larger than that on PLGA-Dex-SMS and PLGA-Com-SMS.

ALP is expressed mainly on cell surfaces or in matrix vesicles. It specifically degrades the organic phosphoesters in bone and cartilage which inhibits cartilage mineralization and promotes calcium deposition in bone (Wang et al., 2005). Type I collagen constitutes a remarkable majority (over 90%) of bony structural proteins and it takes major responsibility for the tensile strength of bone tissue and also provides templates for biomineralization (Blumberg et al., 1997). In this study, we found that dexamethasone from PLGA-Dex-SMS promoted ALP mRNA expression and ALP secretion, which is in agreement with the reports by (Jaiswal et al., 1997) who demonstrated that dexamethasone stimulated the ALP activity of hMSC. The effect of dexamethasone on type I collagen expression is controversial, and both positive and negative effects have been reported (Jabbarzadeh et al., 2007). Our study indicates a significantly higher expression of type I collagen for cells on dexamethasone-containing scaffolds when compared with dexamethasone-free scaffolds. AA can stimulate the secretion and processing of type I procollagen component and enhance procollagen synthesis and gene expression (D'ippolito et al., 2002). AA has been shown to induce the synthesis of type I collagen in osteoblasts and chondrocytes (Owen et al., 1990; Blumberg et al., 1997). It has been reported (Fratzl-Zelman et al., 1998) that β-GP when added to osteoblast cultures promotes *in vitro* mineralization in tissue and cell culture (Zhang et al., 2003). As shown in Figs. 6 and 7, PLGA-Com-SMS displayed remarkably enhanced type I collagen mRNA expression and type I collagen secretion.

OC is an important late bone marker that regulates the formation and growth of bone minerals and therefore indicates the finalization of osteogenesis (Shi et al., 2009d). The expression levels of OC are thus associated with mineralization in a variety of

cell types. Beresford et al. (Beresford et al., 1994) demonstrated that dexamethasone treated hMSCs display undetectable OC mRNA expression. In this work, weak OC mRNA expression (Fig. 6) and OC secretion (Fig. 7) were detected on PLGA-Dex-SMS. Contrastively, positive expression of OC and overwhelmingly enhanced OC secretion and calcium deposition were observed on PLGA-Com-SMS, which may be attributed to AA-dependent ECM synthesis and β-GP inducing mineralization.

In summary, osteogenic drugs loaded PLGA-based microsphere scaffolds were fabricated by both single and double emulsion techniques in this study. Drugs released from the scaffolds showed successfully induced osteogenic differentiation of hMSCs on the scaffolds *in vitro*. Therefore, as favorable drug vehicles, PLGA-based microsphere scaffolds show promising potential for bone repair applications.

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