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Evaluation of bone matrix and demineralized bone matrix incorporated PLGA matrices for bone repair

A. Champa Jayasuriya · Nabil A. Ebraheim

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Abstract The aim of this study was to evaluate the composite matrices prepared using Poly(lactic-co-glycolic acid)- PLGA (85:15) by incorporating human bone matrix (BM) powder or demineralized bone matrix (DBM) powder with the weight ratio of polymer: BM or DBM (75:25) to apply for bone repair. Murine Bone Marrow Stromal Cell (BMSC) attachment was studied with different time points at 30 min, 1 h, 2 h, 4 h, and 6 h for BM/PLGA, DBM/PLGA and PLGA control matrices. All types of matrices were linearly increased the BMSC attachment with the increase of time. Significantly higher number of BMSCs was attached to the both BM/PLGA and DBM/PLGA matrices after 2 h compared to the controls. If BM or DBM is incorporated into biodegradable PLGA matrices and cultured with BMSCs, those composite matrices could be potentially used for bone tissue engineering applications. In addition, particle migration and handling difficulties in DBM powder in clinical applications eliminate using a PLGA matrix. Furthermore, we have observed that DBM/PLGA matrices were structurally stronger compared to the BM/PLGA or control PLGA matrices when they exposed to physiological environment for 72 days.

1 Introduction

Mineralized and demineralized freeze-dried bone allografts have been used for the regeneration of orthopaedic [1–6], dental [7, 8], and craniofacial [9, 10], applications such as bone injuries, defects and irregularities in humans. These allografts provide osteogenic components such as a mixture of proteins and growth factors including bone morphogenetic proteins (BMPs), a powerful regulator for bone formation [11, 12]. Mineralized allograft consists mineral, hydroxyapatite which provides osteoconductive properties, and mechanical strength to the bone to bear the loads.

Demineralized bone matrix (DBM) is the least immunologic of the allograft bone types [9] and extracted by removing the mineral in the bones using acids, allowing the organic and protein constituents to remain [9]. Urist demonstrated cartilage and heterotopic bone formation after the implantation of allogenic DBM at intra-muscular sites in rodents. He hypothesized that the presence of a protein (introduced as BMPs) was responsible for inducing cartilage and bone formation [11–13]. This hypothesis suggested that BMP is released from a macromolecular aggregate of noncollageneous proteins in the process of normal bone turnover or in response to implantation. Reddi et al. has been reported that bone induction is a sequential biological chain reaction that consists of chemotaxis and proliferation of mesenchymal cells and differentiation of bone [14]. Undifferentiated mesenchymal cells migrate to the surface of the matrix particles by chemotaxis, increase their number by mitosis, and differentiate, first into chondrocytes producing cartilage and later into osteoblasts producing bone. Some investigators have reported that native insoluble BMP and noncollageneous protein induced differentiation of mesenchymal cells into chondrocytes in vitro [5, 15].

More extensive use of DBM powder in clinical applications is limited due to several reasons including difficulties in handling and concerns about particle migration [5, 16]. Even though there are several forms of demineralized

A. Champa Jayasuriya (✉) · N. A. Ebraheim
Department of Orthopaedics, University of Toledo, Health Science Campus, Mail Stop 1094, 3065 Arlington Avenue, Toledo, OH 43614-5807, USA
e-mail: a.jayasuriya@utoledo.edu

allograft bone products are commercially available, clinical failure in orthopaedic applications are reported 50% [17]. Most of commercially available DBM forms use liquid such as glycerol, and localized release of osteogenic components may not expect because of their rapid diffusional excretion from the site [18]. It is therefore necessary to contain the osteogenic components in a carrier which does not disperse immediately so that it will have a localized effect at the bone healing site. One possible way for enhancing in vitro efficacy is to achieve its controlled release over an extended time period by incorporating the bone matrix (BM) or DBM in a biodegedable polymer matrix.

Biodegradable Poly(lactic-co-glycolic acid)-PLGA (85:15) co-polymers have been used extensively as sutures, plates, and screws for repair of bone fractures [19, 20], drug delivery systems and templates for tissue engineering applications [21–23]. PLGA co-polymers hydrolytically degrade into metabolic products (lactic, glycolic acid) which are incorporated into metabolic pathways (tricarboxylic acid cycle) and eventually excreted as CO_2 and H_2O [24]. Therefore, PLGA is biodegradable, biocompatible, osteoconductive and United States Food and Drug Administration approved polymer material for clinical applications. The biodegradation level of PLGA can be regulated by choosing appropriate amount of copolymer ratio. In addition, PLGA copolymers have been shown to support osteoblast attachment, growth and function [25, 26].

In the present study, we hypothesized that incorporation of BM and DBM powder into PLGA could be avoided the particle migration and handling difficulties in clinical applications. We have also evaluated the murine bone marrow stromal cell (BMSC) attachment in BM/PLGA and DBM/PLGA matrices in vitro for potential use in bone tissue engineering applications.

2 Materials and methods

2.1 Preparation of DBM

Fresh freeze-dried human cadaver bone specimens were used to obtain BM and DBM powder which were prepared according to protocol published by Glowacki & Mulliken and briefly as follows [9]. Bones were cleaned for muscle, cartilage, connective tissue and then hammered to get small pieces. The pieces of bone were cleaned free of marrow and adherent soft tissues and washed thoroughly with cold, deionized water. The extraction of cleaned tissue was carried out with several changes of absolute ethanol for at least 1 h. The DBM particles were dehydrated using anhydrous ethyl ether in a fume hood for 1 h and then stored at room temperature. The dehydrated material was pulverized in a freezer impact mill (Spec, New Jersey). The pulverized BM powder was demineralized using 0.5 M HCl for 3 h at room temperature. The acid and free minerals were washed away with cold, deionized water. The DBM particles were extracted with changes in absolute ethanol for 1 h. The material was then extracted in a fume hood with changes of anhydrous ethylether for 1 h and left in the hood overnight in order to evaporate the remaining ethylether.

2.2 Incorporation of BM/DBM into PLGA films

PLGA (85/15) copolymer Medisorb Alkerenes® amorphous with glass transition temperature (Tg = 55°C) was dissolved in chloroform to fabricate films. After drying thoroughly more than 24 h under air circulation hood followed by vacuum oven, smooth transparent films were peeled off from the petri dishes and kept in the desiccator until use. The PLGA films were used as controls.

BM or DBM particles were added with the weight ratio of PLGA:BM/DBM 75:25 while PLGA in the soluble state. The BM particles were approximately in the range of 25–50 μm. The BM or DBM in PLGA suspension was sonicated 10 min to disperse the particulates. Then BM/DBM incorporated PLGA films were prepared casting suspension into the Petri dish and final thickness of all matrices were approximately 80 μm.

2.3 Morphology of matrices—scanning electron microcopy (SEM)

Images of morphology in all types of matrices at different time points were obtained using a Hitachi S3200 SEM operating with 20 kV accelerating voltage under high vacuum. Conventional secondary electron scintillator detector was used with tungsten filament. Matrices were coated with a 20 nm gold layer using a Denton vacuum model Desk II sputter coater. For each matrix at each time point, two matrices ($n = 2$) were used for SEM analysis and observed at low magnification (50×). Then the one of the most similar area in two matrices at each time point was selected and imaged with higher magnification (200×). Therefore, SEM images represent the statistically meaningful results. However, at 72 days, PLGA control and BM/PLGA samples were partially broken and the large pieces of matrices that remained were used for the SEM analysis.

2.4 Evidence of having osteogenic and osteoconductive components—fourier transform infrared (FTIR) spectroscopy

A piece of film (~20 mg) was ground into a fine powder of BM or DBM and blended with KBr and then pressed to

get a pellet, which was used for FTIR measurements. The same method was used to obtain FTIR spectra for BM/PLGA and DBM/PLGA matrices.

2.5 BMSC isolation and culture

The C57/BL-6 strain, 6 weeks old, male mice were purchased from the Charles Rivers Laboratory, Wilmington, MA. The mice were housed in the animal care facilities of University of Toledo Health Science Campus. One mouse was euthanized by CO_2 inhalation performed according to the American Veterinary Medical Association (AVMA) panel on euthanasia and the University of Toledo guidelines. Murine BMSCs were isolated by flushing the femurs from the mouse using α-Minimum Essential Medium (MEM) at 1000 rpm for 10 min and the cell pellet was resuspended in fresh α-MEM. All preparations were pipetted repeatedly to break up cell aggregates. Cells were then cultured at 37°C, 95% humidity and 5% CO_2 in flasks containing 10 ml of growth medium with α-MEM, 10% volume fraction of fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. The resulting adherent cells were harvested as follows: cells were washed twice with Hank's balanced solution, treated with two consecutive applications of trypsin EDTA for 3–5 min each at 37°C, and washed with growth medium. BMSCs were maintained in growth medium with media changes every 3–4 days.

2.6 BMSC attachment

PLGA, BM/PLGA and DBM/PLGA solutions were casted into glass coverslips (circular shape with diameter 12 mm) and used for cellular experiments. All matrices were sterilized applying UV radiation for 15 min under the cell culture laminar hood. If PLGA films were subjected for extended time for UV radiation the sample surface would be damaged. Therefore, UV radiation was applied only 15 min to samples. Since Ethylene oxide is highly toxic and γ-irradiation would be changed the properties of PLGA, we did not use those two methods to sterilize the samples. Murine BMSCs from first passage was seeded into PLGA, BM/PLGA and DBM/PLGA matrices in the 24 well plates with the cell culture media containing α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cell density used for experiments was 50,000 cells/ml and 0.5 ml of media with cells pipetted into 24 well plates. Cell attachment was tested for each time point at 30 min, 1 h, 2 h, 4 h, and 6 h after washing unattached cells with phosphate buffered saline (PBS) and then attached cells were tripsinized and counted using a Coulter counter.

2.7 Statistics

The values on each graph represent mean and standard deviation. The number of attached cells and mass of the matrices were statistically analyzed with 1-way ANOVA for effects of matrice type and time followed by Tukey post hoc multiple comparisons using Sigmastat software version 13.0. Values of $P < 0.05$ were considered as statistically significant.

3 Results

3.1 Evidence of osteogenic and osteoconductive components-FTIR

For the BM powder sample IR peaks appeared at 1645 cm^{-1} (v_3) for Amide I (C=O stretching) and at 1536 cm^{-1} (v_3) for Amide II (N–H bending) respectively, due to the osteogenic components in the bone matrix. The peaks at 1022 (v_3), 602 (v_4), and 560 cm^{-1} (v_4) were characteristics peaks for PO_4^{3-} (Fig. 1b). DBM powder sample also exhibited the C=O stretching and N–H bending peaks at 1648 and 1527 cm^{-1} (v_3) (Fig. 1a). BM/PLGA and DBM/PLGA matrices were shown evidence of having osteogenic components similar to BM and DBM powder appearing C=O stretching at 1648, 1646 and N–H bending bands at 1537, 1538 cm^{-1} respectively (Fig. 1c, d). Figure 1c and d exhibited the characteristic IR peaks for PLGA: at 1000–1300 cm^{-1} for ester bond, at 1737 cm^{-1} for carbonyl peak (C=O stretching), and at 2878–2994 cm^{-1} for methyl groups. However, ester bond peaks in the range of 1000–1300 cm^{-1} in Figure 1c and d appeared with poor resolution.

3.2 BMSC attachment on matrices

All types of matrices, just after preparation, without subjecting to any degradation, were linearly increased the BMSC attachment with the increase of time. The BMSCs grown in wells containing BM/PLGA and DBM/PLGA demonstrated significantly higher BMSC attachment at 2 h, 4 h, and 6 h compared to cells grown in wells with controls ($P < 0.05$) (Fig. 2). However, there were no significant difference of cell attachment was observed for the DBM/PLGA matrices and BM/PLGA matrices at all time points except 2 h ($P < 0.05$). At 6 h, the attached number of BMSCs were approximately 5000, 7500, and 7000 for the control, DBM/PLGA, and BM/PLGA samples, respectively.

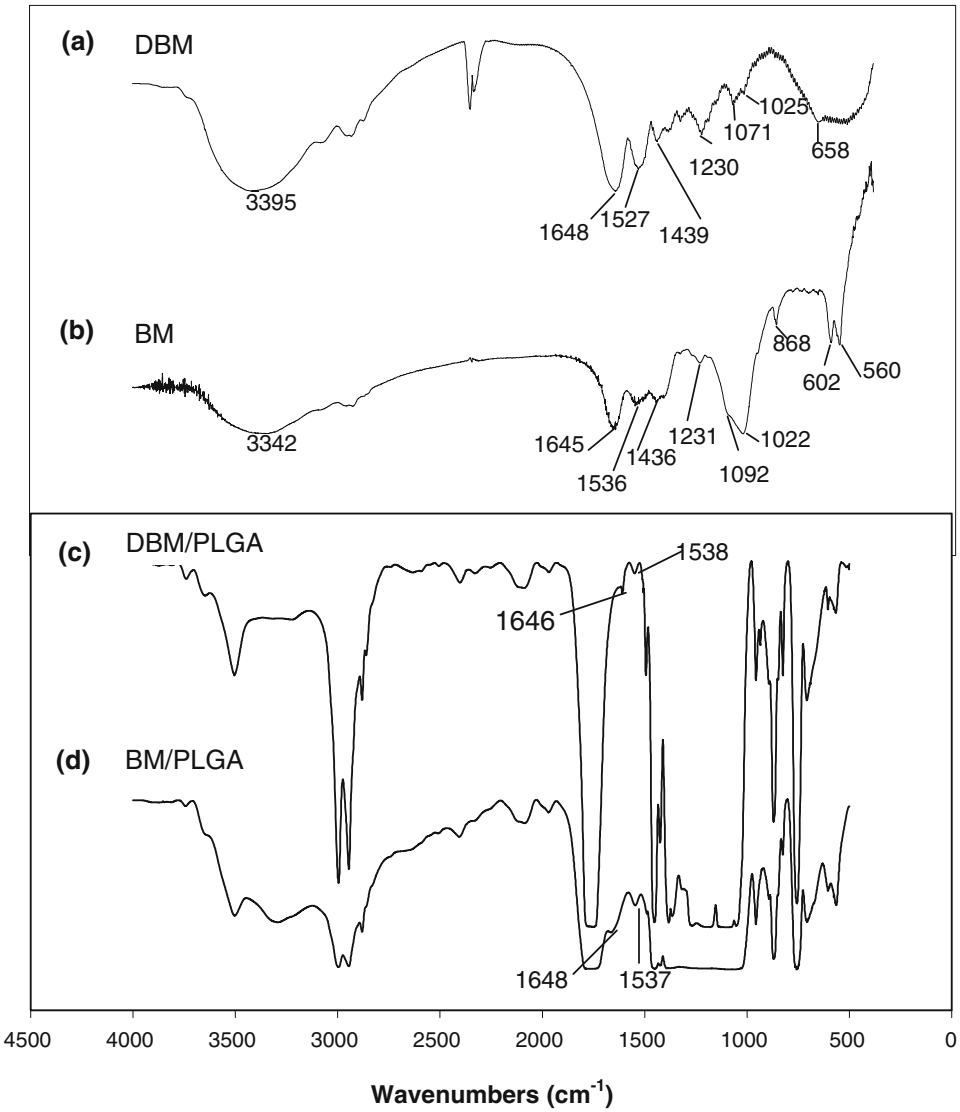

Fig. 1 FTIR spectra for **a** DBM powder; **b** BM powder; **c** DBM/PLGA; **d** BM/PLGA

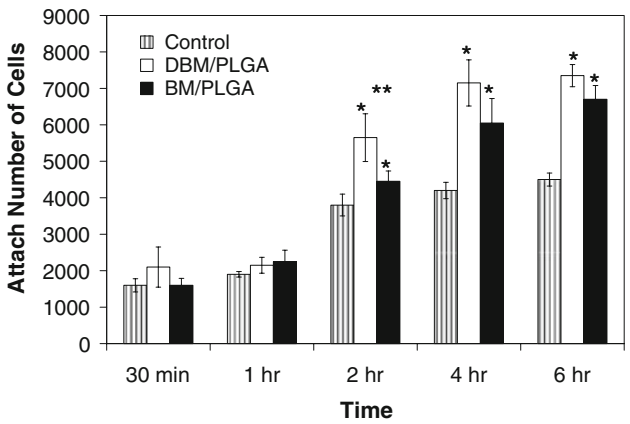

Fig. 2 BMSC attachment on PLGA (control), DBM/PLGA and BM/PLGA matrices. *Indicates significantly higher BMSC attachment on DBM/PLGA and BM/PLGA matrices at 2 h, 4 h, and 6 h compared to controls ($P < 0.05$). **Indicates significant difference of cell attachment on DBM/PLGA and BM/PLGA at 2 h ($P < 0.05$)

3.3 Morphology changes-SEM

The SEM images of morphology for PLGA, BM/PLGA and DBM/PLGA matrices at 0, 24, 48, and 72 days are shown in Figs. 3, 4, and 5, respectively. Control PLGA is shown smooth surface at 0 weeks, then gradually degraded features appeared at 24 and 48 days, and circular shape of pores appeared due to the further degradation at 72 days (Fig. 3). BM powder was distributed homogeneously throughout the PLGA matrix at 0 weeks (Fig. 4a). PLGA matrix seems to be slightly ruptured exposing to PBS and BM particles can be observed in the surface of BM/PLGA matrix at 24 days (Fig. 4b) and further degraded surface appeared at 48 days (Fig. 4c). There were more BM particles observed in the surface of BM/PLGA with less of polymer matrix due to the degradation at day 72 (Fig. 4d), compared with days 24 and 48. At 24 day, there were few holes presented due to the degradation of PLGA/DBM (Fig. 5b) but it was unlike to the

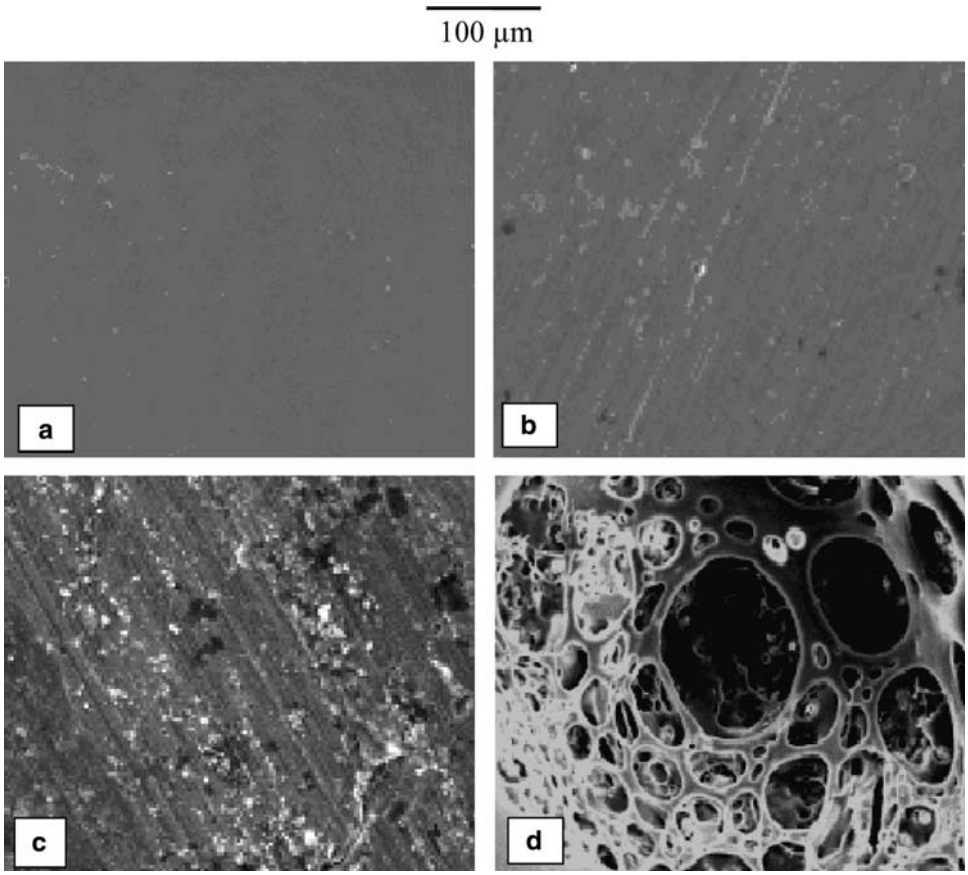

Fig. 3 SEM images of PLGA controls at different time points exposed to PBS **a** 0 days; **b** 24 days; **c** 48 days; **d** 72 days

BM/PLGA at day 24. More degraded features were appeared at day 48 with holes in the DBM/PLGA matrices. A day 72, DBM/PLGA matrices exhibit more degradation features compared with day 24 and day 48.

3.4 Mass loss

The dry mass of all types of matrices was measured before and after placement in PBS at each time point. Normalized mass loss was calculated (4 matrices at each time) from the ratio of mass of matrices after exposing to PBS at each time point to their initial mass. Normalized mass loss for each type of films is shown in Fig. 6. BM/PLGA matrices start to exhibit higher mass loss relative to other two types of matrices. The significant mass loss was not observed for DBM/PLGA matrices throughout the period exposure to PBS. PLGA control films start to loss their mass significantly after 60 days and then loss all most all mass around 80 days, but DBM/PLGA matrices loss their mass less than 20% even at 80 days.

4 Discussion

In the present study, we successfully prepared the BM, DBM incorporated PLGA composite matrices using a simple method. There are several advantages that can be derived from these composite matrices when they are applied to treat the bone: (1) The DBM or BM particle migration, immediate dispersion with blood or handling difficulties during the clinical applications can be avoided; (2) Since the PLGA matrix is completely degradable, it could be implanted close to the site where it is needed, such as in the bone to treat bone fracture; (3) Osteogenic and osteoconductive components in the matrices can be released in a controllable manner when patients are treated for long-term bone fractures; (4) Another unique feature of these matrices is that it can be fabricated with different sizes and variety of shapes according to the target site because these PLGA polymers are very flexible.

We evaluated in vitro BMSC attachment at different time points on control, DBM/PLGA, and BM/PLGA, matrices. Both DBM/PLGA and BM/PLGA matrices exhibit statistically significant higher amount of BMSC attachment after 2 h and beyond compared to PLGA controls. BMSCs seem to be favorable to attach DBM/PLGA or BM/PLGA matrices than the control probably due to the existence of osteoconductive and osteoinductive components in BM or DBM. Some studies have shown induce function of BMSCs which seeded in the matrices containing DBM or partially DBM [27, 28]. Their results demonstrate that partially demineralized bone can be

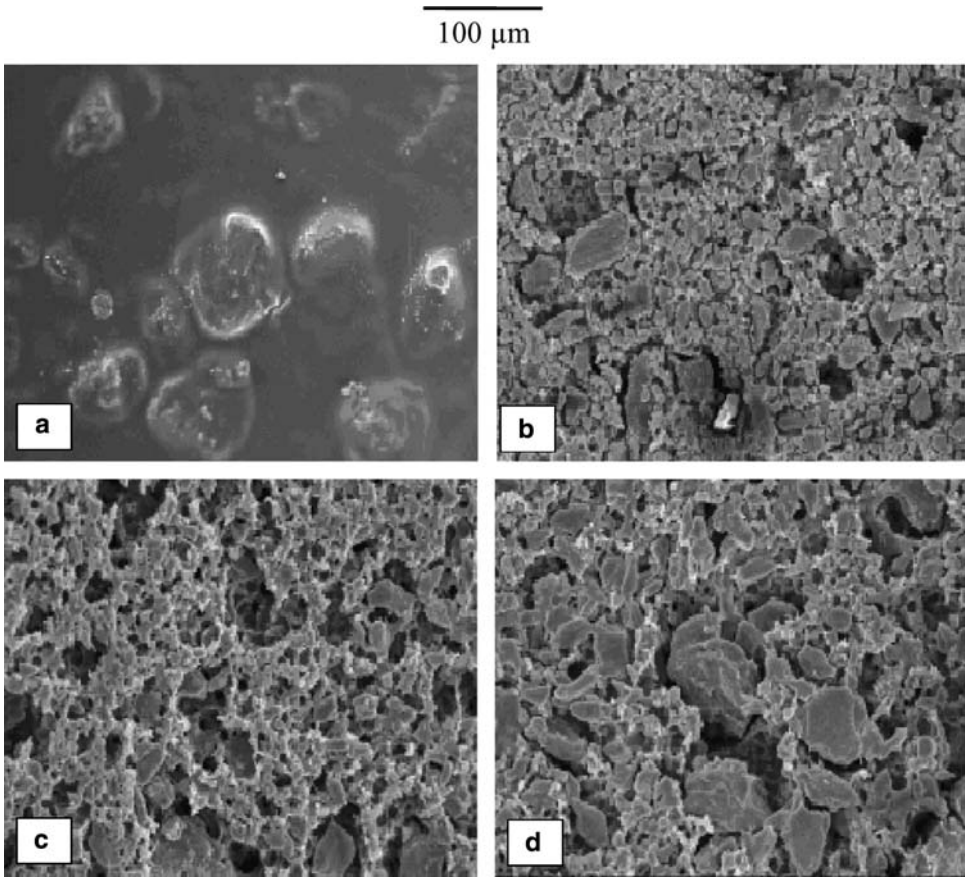

Fig. 4 SEM images of BM/PLGA controls at different time points exposed to PBS **a** 0 days; **b** 24 days; **c** 48 days; **d** 72 days

successfully used with human BMSCs to support osteogenic differentiation in vitro [27]. The expression of osteocalcin increased up to 5-fold in DBM matrices compared to the values of the ceramic matrices after 21 days [28]. This difference has been explained that DBM consist of collagen, a variety of adhered growth factors such as BMPs or TGF-β. Another study has shown that active BMP in DBM can stimulate osteogenetic differentiation of premyoblasts by inducing (Alkaline Phosphatase) ALP activity [29]. Similar to our BMSCs attachment on BM/PLGA and DBM/PLGA, we speculate that BMSCs seeded BM/PLGA and DBM/PLGA matrices will be significantly enhanced the osteoblast gene expression markers and secretion of extracellular matrix, compared to PLGA control samples, since those samples possess osteoconductive and osteoinductive components.

According to SEM images in Figures 3, 4, and 5, it seems that both BM/PLGA and PLGA matrices degrade faster than DBM/PLGA matrices. Agreeing with this result, higher mass loss (Fig. 6) was observed for BM/PLGA followed by PLGA matrices during the incubation. The mineral particle leached out from the BM/PLGA matrices (Fig. 4) and therefore, higher mass loss was observed in BM/PLGA matrices. Degradation of PLGA is a hydrolytic process. Cleavage of an ester bond yields a carboxyl end group and a hydroxyl group [30–32]. Degradation of semicrystalline polyesters in aqueous media occurs in two stages [32]. The first stage consists of water diffusion into the amorphous regions with random hydrolytic scission of ester bonds. The second stage starts when most of the amorphous regions are degraded. The hydrolytic attack then proceeds from the edge towards the center of the crystalline domains. Mass loss of DBM/PLGA depends on the release of water-soluble oligomeric by products as well as release of osteogenic components during the incubation period. The DBM/PLGA matrix consists of osteogenic components and it can affect the local environment inside polymer matrix such as pH and hydration behavior. Due to these reasons, DBM/PLGA matrix degradation seems to be delayed and hence less mass loss was observed during the 80-day incubation period.

5 Conclusions

The composite PLGA matrices prepared in this study using DBM or BM can be potentially applied for bone repair applications in clinical orthopaedics and craniofacial tissue

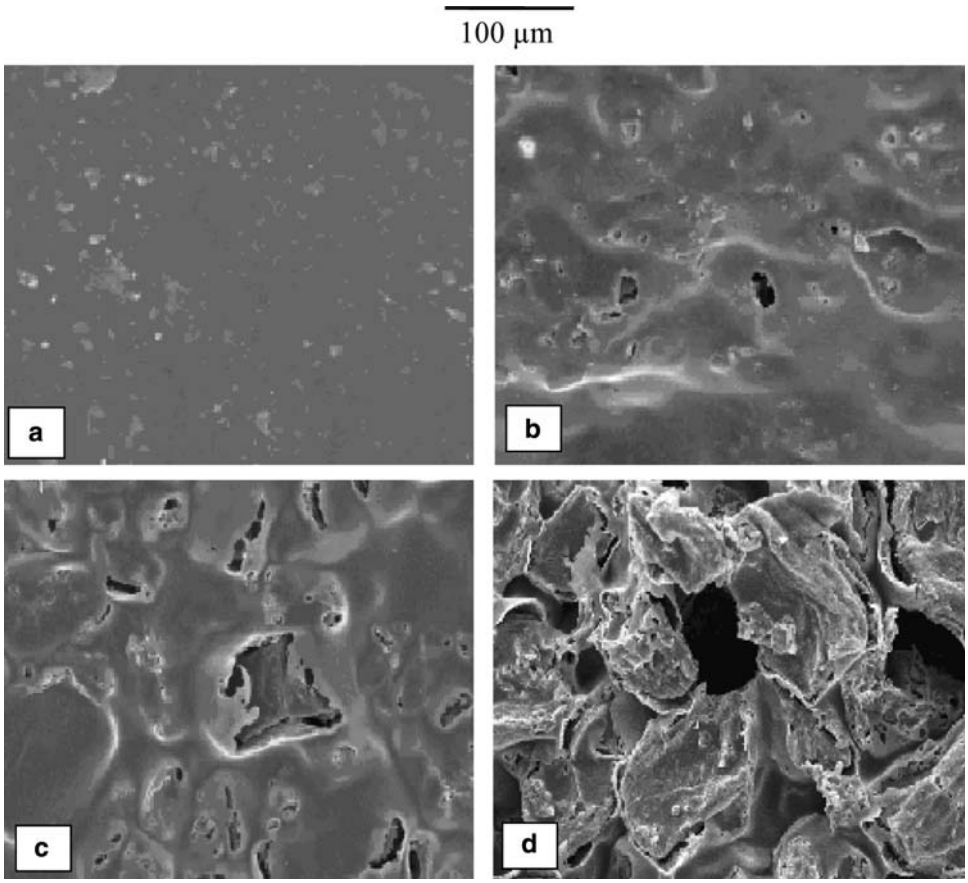

Fig. 5 SEM images of DBM/PLG controls at different time points exposed to PBS **a** 0 days; **b** 24 days; **c** 48 days; **d** 72 days

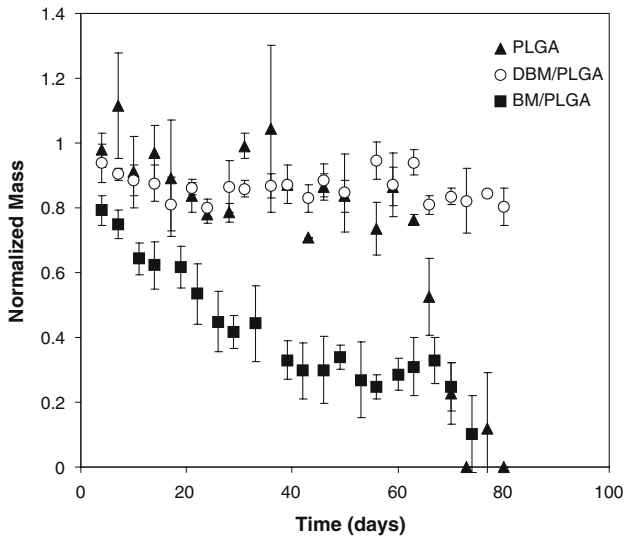

Fig. 6 Normalized mass loss for PLGA, DBM/PLGA and BM/PLGA matrices as a function of time exposed to PBS

regeneration. These novel composite formulations may reduce the drawbacks caused by BM or DBM particles when used in the clinical treatment of critical size bone defects.

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