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Osteogenic differentiation of mesenchymal stem cells on a poly (octanediol citrate)/ bioglass composite scaffold in vitro

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ABSTRACT: This study investigated the effect of composite scaffolds composed of poly (octanediol citrate) (POC) and a bioactive glass (composition, $48\%\text{SiO}_2-12\%\text{CaO}-32\%\text{ZnO}-8\%\text{Ga}_2\text{O}_3$) on the growth and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs). All the scaffolds, regardless of the amount of bioglass incorporation, were able to support the growth of hBMSCs and guide their osteogenic differentiation without osteogenic media stimulation. The expression of bone-associated genes (run-related transcription factor 2, type I collagen, bone morphogenetic protein 2, osteonectin and osteocalcin) was significantly increased by a culture time of up to 2 weeks, particularly for the composite scaffold loaded with 10% bioactive glass. The composite scaffolds significantly stimulated alkaline phosphatase (ALP) activity compared to the pure POC scaffold. Cellular mineralization of the secreted extracellular matrix illustrated a higher calcium level on the composites than on the pure POC and increased with culture time. These results suggest that composite scaffolds of POC and a bioactive glass can provide favourable
conditions for osteogenic differentiation of hBMSCs and can potentially be used to induce bone healing and regeneration.

**Keywords:** Composite scaffold, bioactive glass, human bone marrow mesenchymal stem cells, osteogenic differentiation, bone tissue engineering

1. **Introduction**

Bone is one of the most commonly transplanted tissues of the body with over 2 million grafting procedures annually worldwide [1]. However, skeletal defects caused by tumors or traumatic bone loss present the need for complex treatment strategies [2], often requiring the use of autografts, allografts, or metallic and ceramic implants, each of which has its own disadvantages such as donor site morbidity, disease transmission, and mismatch of mechanical properties with the native bone [3]. Recently, tissue engineering has emerged as an alternative approach to create *de novo* tissue by growing cells on 3D scaffolding [4]. An ideal bone graft substitute or tissue scaffold should provide the necessary support for the cells to attach, proliferate and facilitate ingrowth [5]. In parallel with tissue formation, an ideal scaffold should degrade and create open space for new bone formation until regeneration is achieved. Accordingly, biodegradable scaffolds have the potential to reduce the number of surgeries, since there is no need for an additional operation to remove the implant.

A crucial factor identified in the failure of many tissue-engineered constructs is inadequate tissue regeneration around the biomaterial immediately after implantation [6]. Since the interaction of cells with biomaterials is a fundamental parameter in the evaluation of a scaffold, a number of recent contributions to the literature have focused on the design and development of biomaterial structures that facilitate favourable interactions and augment tissue regeneration. *In vivo* bone formation involves osteogenic reparative cells originating from mesenchymal stem cells (MSCs) in bone marrow, the presence of a regeneration template, and the provision of regulatory signals [7]. Human mesenchymal stem cells (hMSCs) isolated from bone marrow serve as an ideal cell source for a wide variety of cell-therapy concepts due to their self-renewal ability, multilineage differentiation potential and immunomodulatory properties [8]. They are also capable of secretion of biomolecules such as cytokines, chemokines, growth factors, and extra cellular matrix (ECM) molecules, in a paracrine or even autocrine manner, which influence the surrounding environment to promote angiogenesis, reduce inflammation, and enhance tissue repair [9]. For the application of
hMSCs to bone regeneration, a better understanding of the interactions occurring between hMSCs and biological scaffold material is essential because the interaction at the cell-biomaterial interface plays a major role in the bonding of implant materials to bone.

The choice of scaffold is a critical factor in the development of competent tissues with the desired characteristics [10]. Synthetic biomaterials are now being designed with a combination of both resorbable and bioactive characteristics to stimulate regeneration of living tissue. So far, there is no single biomaterial that is able to satisfy all the requirements for an ideal bone graft driving tissue engineers to create 3D scaffolds made up bioceramic and polymeric materials to facilitate normal bone growth.

Poly (octanediol citrate) (POC) has been investigated as such a scaffold due to its biomimetic viscoelastic properties, linear degradation profile and non-toxic degradation products. The mechanical properties and biodegradation rate of POC can be controlled by altering curing conditions (time and temperature) and the initial monomer molar ratio to mimic the pliancy of certain soft tissues such as blood vessels, urinary bladder smooth muscle and myocardium [11]. POC appears to have good compatibility with a number of cells, including articular chondrocytes, endothelial cells, myoblasts and osteoblasts, without requiring any additional treatment [12-15]. However, poor mechanical properties have retarded its use in load bearing applications driving attempts to manipulate the physicochemical properties of POC by synthesis and fabrication of co-polymers and composites suitable for hard tissue engineering [16-18]. POCs low stiffness makes it a suitable material for accepting a large amount of fillers without the detrimental effect of stress shielding [19]. Several studies have been conducted to develop composites of POC with ceramics such as hydroxyapatite (HA), calcium silicate and bioactive glass in order to improve both mechanical properties and the osteoinductivity [17, 19, 20]. It has been shown that tissue ingrowth into POC-HA is increased compared to ingrowth into poly (L-lactic acid), which highlights an additional benefit of these novel biomaterials. HA exhibits a low dissolution rate, whereas bioactive glasses are more soluble and their degradation products are released into the surrounding environment, potentially inducing bioactivity, depending upon what constitutes those degradation products [21].

Bioactive silicate glasses have achieved great success in clinical applications due to their well-known osteoconductive and osteoinductive properties [21]. For example, ionic dissolution products of bioglasses can up-regulate the expression of genes of osteoblastic cells, which in turn control osteogenesis and promote bone formation [22]. Bioglasses can bond to both hard and soft tissues through rapid formation of hydroxyl carbonate-apatite on
the glass surface upon implantation, which promotes cell migration and differentiation. Bioactive glasses can be doped with therapeutic elements to modulate the desired tissue responses. For instance, the release of magnesium or potassium ions can increase bioactivity, silver, zinc (Zn) or gallium (Ga) ions can impart antibacterial properties, and strontium ions can enhance bone cell responses [23]. Although there have been many studies of the interaction of composite scaffolds with MSCs, to our knowledge there have been no studies of stem cell response to composites of POC and a bioactive glass.

Recently, we have shown that a POC scaffold loaded by a bioactive glass containing Zn$^{2+}$ and Ga$^{3+}$ can stimulate collagen type I and III secretion in human osteoblast-like cells as well as imparting effective antibacterial activity against *E. coli* and *S. aureus* bacteria [16, 24]. In the present study, a range of composite scaffolds comprising POC/bioglass were cultured with hBMSC to observe the possible influence of the bioglass phase on *in vitro* osteogenic differentiation potential.

## 2. Materials and methods

### 2.1. Fabrication and characterization of composite scaffold

Synthesis and characterization of POC pre-polymer and fabrication of POC-bioactive glass (0.48SiO$_2$-0.12CaO-0.32ZnO-0.08Ga$_2$O$_3$ molar fraction) composite scaffolds was carried out as previously reported [16]. In brief, POC pre-polymer was synthesized successfully by the polycondensation reaction of high purity citric acid and 1,8-octanediol (1:1 mole ratio), then dissolved in 1,4-dioxane and mixed with various amount of bioactive glass (≤45 µm) to obtain composites with 10, 20 and 30 wt% bioactive glass, named hereafter POC-10%BG, POC-20%BG and POC-30%BG respectively. After that the solutions were sonicated for 30 mins followed by the addition of 90 wt% of salt porogen (200–300 µm). The resulting materials were post-polymerized at 80 °C for 7 days. Salt in the resulting composites was washed for 4 days in distilled water and subsequently the porous scaffolds were freeze-dried. Disk-shaped scaffolds (6 mm diameter × 3 mm thickness) were punched out for the subsequent experiments. The scaffold morphology was analyzed by a field emission scanning electron microscopy (FESEM: Quanta™ 250 FEG—FEI, USA), at 20 kV. The scaffolds were gold-coated using a 150 rotary-pumped sputter coater (Quorum Technologies).
2.2. Cell culture and seeding

Using standard laboratory protocols, hBMSCs were isolated (ethical approval for human bone marrow collection was obtained from the medical ethics committee of University of Malaya Medical Centre; MECID.NO: 201412-865). The cells were cultured in a medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin (Sigma–Aldrich, USA), and 100 mg/mL streptomycin (Sigma–Aldrich) in tissue culture flasks at 37 °C under a humidified atmosphere of 5% CO₂. When the cells reached near confluence (80%–90%), they were detached by trypsin/EDTA (Cell Applications, San Diego, CA, USA) and then sub-cultured into the next passage.

Prior to cell culture experiments, the samples were sterilized by incubation in 70% ethanol for 3 h and washed with sterile PBS (pH 7.4). After sterilization, samples were washed 3 times in serum-free cell culture medium for 48 h. Then, the desired amount of the cells (5 × 10⁴) in the growth medium was dropped into the scaffold and left for 1 h in the incubator (37°C and 5% CO₂) for cell attachment and then topped up with 1 ml of media.

2.3. Cell adhesion and morphology

Cell attachment and morphology on the scaffolds was observed by a FESEM (Quanta™ 250 FEG—FEI, USA). Cells were cultured for a period of up to 14 days into the scaffolds, fixed with 4% glutaraldehyde in PBS at 4°C overnight and post-fixed in 2% osmium tetroxide. After thorough washing with PBS, the scaffolds were dehydrated in a serial ethanol. Then, the samples were immersed in hexamethyldisilazane (Sigma-Aldrich) twice for 10 min and air-dried at room temperature by keeping the samples in fume hood. The dried samples were sputter coated with gold and observed under FESEM.

2.4. Cell distribution and density

To investigate cell distribution and density within the scaffolds, hBMSCs-laden scaffolds were stained with NucBlue® Live ReadyProbes™ Reagent (Hoechst 33342, Invitrogen, USA) solution; 1 drop of NucBlue per ml of medium (according to the manufacturer's description). After 20 min of incubation at room temperature the samples were analyzed using confocal laser scanning microscopy (CLSM; Leica TCS SP5 II, Leica Microsystems CMS GmbH, Mannheim, Germany) at excitation/emission, 495 nm/515 nm.
2.5. **Cell proliferation**

Cell proliferation was assessed with AlamarBlue® (DAL1025, Invitrogen, Inc.) according to the standard protocol. Before the AlamarBlue® was added, the media was replaced. AlamarBlue® was added to the samples (10% v/v of medium) and incubated at 37 °C in an incubator (with 5% CO₂) for 4 h. The supernatant samples were pipetted into a centrifuge tube and centrifuged at 10,000 rpm. 100 µl Aliquots from each sample were transferred to a 96-well plate, and the fluorescence of AlamarBlue® was measured using a fluorescence plate reader (BioTek epoch) at a wavelength of 570 nm and 600 nm.

2.6. **ALP activity**

The differentiation of the hBMSCs was evaluated by measuring the alkaline phosphatase (ALP) activity using an ALP Colorimetric Assay Kit (Abcam, ab83369) employing p-nitrophenol phosphate as substrate. After culturing for 3, 7 and 14 days, the cells were washed with PBS and trypsinized. The cell pellets were disrupted via a freezing/thawing process. Following that, 30 µl supernatant were added to 50 µl of pNPP ALP substrate solution and incubated at 37 °C for 60 min. Action was then stopped by adding 50 µl of stop solution (3N NaOH) into each well. The activity of ALP in cell lysates measured with a microplate reader at 405 nm.

2.7. **RNA isolation and cDNA synthesis and qPCR microarrays**

At 0, 7, and 14 days, the media was removed from the scaffolds by spinning at 1000g for 10s, followed by washing in sterile PBS and spinning again to remove excess liquid. This washing procedure was repeated three times. After the last spin, cultured scaffolds were incubated in Trizol at room temperature for 3 min to complete homogenization. The RNA-containing Trizol solutions were transferred to individual RNAse-free vials and 200µl chloroform was added to each tube, followed by vortex-mixing. After 2 min incubation on the bench, the samples were centrifuged at 12,000 g, 4 °C for 15 min. The upper aqueous layer was collected and RNA was isolated using the AllPrep DNA/RNA Mini Kit (Qiagen; #80204), according to the manufacturers’ instructions. RNA concentration was determined by measuring absorbance at 260 nm in a spectrophotometer (\(A_{260} = 1\) equals 40 mg/ml) and the purity of RNA was estimated from the ratio of readings at 260 nm and 280 nm.
1 µg total RNA for each sample was used as a template for cDNA synthesis using the QuantiTect Reverse Transcription kit (Qiagen), including a gDNA digestion step. The protocol was 2 min at 42 °C, 15 min at 42 °C, and 3 min at 95 °C. Real-time PCR was performed with 1 µl aliquots of the diluted cDNA in a 25 µl reaction volume using QuantiFast SYBR Green PCR kit (Qiagen) to determine the expression level of the osteogenic associated genes such as runt-related transcription factor 2 (Runx2), collagen type I (COL I), bone morphogenetic protein 2 (BMP2), osteonectin (SPARC), and osteocalcin (BGLAP). GAPDH was used as an internal control. Following Taq Polymerase activation step at 95 °C for 5 min, 40 amplification cycles were carried out by denaturing at 95 °C for 30 s and annealing and extension for 30 s at 60 °C. The reaction was monitored in real-time using a MiniOpticon (BioRad).

Table 1: Primer sequences of RUNX2, COL I, SPARC, BMP2, and BGLAP genes used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer Sequences</th>
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| COL I     | Forward 5'- CCC GCA GGC TCC TCC CAG -3'  
Reverse 5'- AAG CCC GGA TCT GCC CTA TTT AT -3' |
| RUNX2     | Forward5'- CCG CCA TGC ACC ACC ACC T -3'  
Reverse5'- CTG GGC CAC TGC TGA GGA ATT T -3' |
| BMP2      | Forward5'- TGG CCC ACT TGG AGG AGA AAC A -3'  
Reverse5'- CGC TGT TTG TGT TTG GCT TGA CG -3' |
| SPARC     | Forward5'- TTG CAA TGG GCC ACA TAC CT - 3'  
Reverse5'- GGG CCA ATC TCT CCT ACT GC -3' |
| BGLAP     | Forward5'- GGA GGG CAG CGA GGT AGT GAA -3'  
Reverse5'- GCC TCC TGA AAG CCG ATG TGG T -3' |

2.8. Determination of calcium content

The calcium content in the supernatant was analysed using a calcium assay kit (Sigma-Aldrich) according to the manufacturer’s protocol. Briefly, at 3, 7, and 14 days after culture, the culture medium was collected for direct measurement or stored at -20 °C. Calcium reagent working solution was then added to 50 µl of each sample, according to the manufacturer’s instructions. The absorbance of the solution was measured at 575 nm using a UV-VIS spectrophotometer, and calcium content was expressed as µg calcium/flask (n = 3).
2.9. **Statistical analysis**

All data were expressed as mean ± standard deviation and processed statistically by the software of IBM SPSS Statistics for Windows, Version 22. The differences of the data were considered significant when P<0.05.

3. **Results**

3.1. **Characterization of bioactive glass and scaffolds**

FESEM micrographs and EDS spectrum of bioactive glass particles are shown in Fig. 1. FESEM image (Fig. 1a) shows that the morphology of bioactive glass particles used to fabricate composite scaffolds was non-spherical, irregular and angular, with at least one dimension less than 45 µm. Furthermore, the EDS spectrum (Fig. 1b) confirmed that the chemical elements in the bioactive glass are silicon, calcium, zinc, gallium and oxygen. The glassy nature of the bioactive glass particles was indicated by the broad halo depicted in the results of the XRD investigation (Fig. 1c), showing that the material was completely amorphous.
Figure 1: (a) FESEM micrograph showing the irregular shape of the glass particles; (b) EDS spectrum of bioactive glass showing the peaks of Si, Ca, Zn, Ga and O; and (c) XRD pattern of the bioactive glass particles showing the typical amorphous halo.

The porous structure of composite scaffolds was created through a solvent casting-porogen leaching technique. Figure 2 (a and b) shows a macroscopic profile of representative POC/bioglass composite scaffolds and their elastic nature. The plan-view FESEM images of a fabricated composite scaffold (Fig. 2c and d) shows the uniform distribution of pores. The composite scaffolds have a highly porous structure with a porosity of ~90 % and a pore size in the range of 200-300 µm, which is found to be optimum for bone tissue ingrowth [25].
Figure 2: Photographs of representative POC-BG scaffolds: (a) demonstration of elastomeric nature of POC-10%BG scaffold; (b) frontal and cross-sectional view of the POC-30%BG scaffold prepared for cell culture. (c, d) Microstructure of POC-20%BG scaffold observed by FESEM at low (50 x) and high (300 x) magnifications.

3.2. hBMSCs adhesion and proliferation

FESEM was used to study the cell attachment, morphology and spreading of hBMSCs on the prepared scaffolds (Fig. 3). After 14 days, the cells were shown to be viable in POC-BG and POC alone. The cells were well-attached to the materials with different morphological characteristics. The cells had flattened morphology on the pure POC and spherical morphology on the POC-BG scaffolds, indicating both differentiated and undifferentiated states. The attached cells showed a round morphology with an increase in the bioactive glass concentration. Although the cells were well-attached to all the scaffolds, fewer cells were observed in POC-20%BG and POC-30%BG, in comparison to POC-10%BG and pure POC scaffolds.

Fluorescence microscopy was further used to study the attachment and distribution of hBMSCs using the nucleic acid staining dye Hoechst (Fig. 3). Blue stained cells were observed on all the scaffolds, indicating that the composition of the scaffolds provided a physiological environment for cell attachment. However, cell density was higher in POC-10%BG than in POC-20%BG and POC-30%BG.
Figure 3: FESEM micrographs of hBMSCs for 2 weeks on pure POC; POC-10%BG; POC-20%BG; and POC-30%BG scaffolds at various magnifications (a, b: 100x), (e, f: 500x), (c, d: 1500x), and (g, h: 3500x). (i-l) Fluorescence microscopy of the cells attached to the scaffolds on day 14 – Hoechst’s staining.

3.3. Cell proliferation

Quantitative in vitro growth of hBMSCs was assessed through the AlamarBlue® assay, as shown in Fig. 4. No statistical difference was observed in the proliferation of hBMSCs on the POC-BG composite scaffolds and the pure POC scaffold after 1 and 3 days of cell culture. Following 7 days of cell culture, the relative proliferation was found to be statistically greater on the POC samples compared to POC-20%BG and POC-30%BG. At each time point, no significant differences were observed in the cell proliferation between POC and POC-10%BG. The highest level of cell proliferation was measured on the POC scaffold.
3.4. ALP activity

ALP activity (Fig. 5) was significantly higher on the POC-BG composite scaffolds in comparison to the pure POC scaffold on days 3 and 7. This is due to the presence of the bioactive glass, which stimulates osteogenic differentiation. ALP activity peaked at day 7 and decreased thereafter. However, no significant difference was observed between POC-10%BG, POC-20%BG and POC-30%BG at day 14. ALP activity was the highest on POC-10%BG at all time points. ALP activity on the pure POC scaffold remained almost constant over culture time.
Figure 5: ALP activity of the hBMSCs on the scaffolds was measured with the pNPP assay at 3, 7, and 14 days of culture; (*P< 0.05) significantly different in comparison to respective pure POC.

3.5. Quantitative RT-PCR

The differentiation of hBMSCs toward an osteogenic phenotype was further analyzed by RT-PCR. The analysis indicates the expression profiles of a series of specific genes that are associated with osteogenesis; namely, RUNX2, COL I, BMP2, SPARC, and BGLAP in 14 days culture period (Fig. 6). The mRNA levels of all the selected genes increased for up to 14 days. For all markers and time points, the POC-10%BG scaffold yielded higher gene expressions than other scaffolds. However, pure POC expressed a significantly higher level of BGLAP (the late osteogenic marker), in comparison to POC-20%BG and POC-30%BG at day 7 and day 14.
Figure 6: RT-PCR results showing transcript levels associated to osteoblastic marker expression of RUNX2, COL I, BMP2, SPARC, and BGLAP at 7 and 14 days after hBMSCs growth on the scaffolds. Data have been normalized to the gene expression levels on day 0; (*P < 0.05) significantly different in comparison to respective pure POC.

3.6. Calcium content

Cellular mineralization on the scaffolds was determined by measuring the Ca content produced by the cells during 14 days culture, as shown in Fig. 7. The results indicated that Ca had been increasing during the culture period. Ca concentration was higher in POC-BG composite scaffold as compared to the pure polymer. However, no significant difference was observed between the three composite scaffolds on days 3 and 7. Furthermore, when comparing the composite scaffolds, the POC-10%BG had significantly higher Ca deposition in the cell construct at day 14.

Figure 7: Calcium content measurements of hBMSCs on the scaffolds during culture for 14 days; (*P < 0.05) significantly different in comparison to respective pure POC.

4. Discussion

It has been previously reported that the delivery of stem cells on a polymer scaffold for the purpose of filling large bony defects resulted in improved bone formation and enhanced mechanical properties, compared to treatment with the scaffold alone [26]. However, transplanted cells often fail to promote the repair of the host tissue, not due to
immune rejection, but rather because of poor scaffold selection leading to an unfavourable delivery. Our study sought to address this issue by fabrication of composite scaffolds made of POC and a bioactive glass [24] and evaluating their capability to support hBMSCs attachment, proliferation and differentiation. The results suggest that scaffold composition plays an essential role in growth and osteogenic differentiation of hBMSCs. The scaffolds were shown to be flexible, suggesting their possible applicability as a bone graft, while allowing a great deal of customization in the hands of the surgeon [27]. Adhesion and morphology of hBMSCs on the materials was observed by FESEM. The evaluation showed that the hBMSCs were well-attached to the composite scaffolds and remained in more-or-less round morphology. The morphology of cells attached to the scaffolds depended on the bioactive glass concentration, and the cell shape became more round with an increase in the bioactive glass content, while on the pure polymer assumed more flattened. This is in agreement with previous studies which demonstrated that cells keep their rounded morphology in the presence of bioactive glasses and during osteogenic differentiation [28, 29]. The results presented in this study indicate the excellent biocompatibility of the scaffolds. It was found that there is no significant difference between cell proliferation on POC and POC-10%BG after 14 days of culture. However, composite scaffolds with 20 or 30% bioactive glass were shown to have very low cytotoxicity. The improved cytocompatibility of the composite scaffold loaded with 10% bioactive glass compared with higher percentages may be attributed to the smaller concentration of ionic dissolution products (Zn$^{2+}$ and Ga$^{3+}$) released from the bioactive glass phase (as shown by the results of our previous study [24]).

It is most likely that the ions released from the bioactive glass phase during leaching and soaking of culture scaffolds had reabsorbed on the composites, leading to modification of scaffold surface properties. Our previous study showed that Zn$^{2+}$ and Ga$^{3+}$ released and reabsorbed on the material itself and its surroundings after soaking in PBS, which confirms the above statement [24]. Salih et al. also found that doping of ZnO to phosphate glasses (P$_2$O$_5$-CaO-Na$_2$O) can increase attachment and proliferation of human osteoblastic cells as compared to Zn-free glass even though the cells did not spread and maintained a round morphology [29]. Accordingly, they have suggested that the addition of certain amount of Zn does not have a detrimental effect on osteoblast responses, and that the cells can still attach which further creates a necessity for surface modification to allow cell spreading [29]. In the current study, the round-shaped morphology of cells on the composite scaffolds could be attributed to the differentiation of hBMSCs towards osteoblast-like cells [30, 31].
Cellular differentiation into osteoblasts was highly affected by the bioactive glass concentration. ALP is considered as a marker of early osteoblastic differentiation and commitment of MSCs toward the osteogenic lineage [32]. ALP activity was highly influenced by the bioactive glass addition so that the entire composites showed a significantly higher ALP activity in respect to pure POC. ALP activity was the highest for the POC scaffold incorporating 10% bioactive glass and after 2 weeks of culture. Since ALP can be expressed by other differentiated cells, it is essential to study other key markers of osteogenic differentiation. Accordingly, we have quantitatively evaluated the relative mRNA expression levels of the early osteogenic markers RUNX2, BMP2, COL I, and SPARC, and the osteogenic late stage marker BGLAP. RUNX2 is considered as a master gene involved in the osteoblast phenotype induction. It is a key regulator of osteogenesis and promotes an upregulation of ALP, BGLAP, osteopontin and bone sialoprotein [33]. COL I is the major protein of bone matrix and is essential for acceleration of osteogenic differentiation, matrix maturation and mineralization [34]. SPARC is a glycoprotein in the bone that binds to both calcium and collagen. It is secreted by osteoblasts during bone formation, thus initiating mineralization and promoting mineral crystal formation [35]. BMP is a naturally occurring protein found in human bone which plays an essential role in angiogenesis and vascularisation of the peristem [36]. BGLAP is one of the osteoblast specific genes and is a noncollagen matrix protein which is closely related to calcification of ECM [37]. The results presented in this study demonstrated that the hBMSCs grown on the POC-BG composite scaffolds expressed RUNX2, COL I, SPARC, BSP2, and BGLAP in the absence of osteogenic media (Fig. 6). Our findings indicated that POC-10%BG significantly up-regulated both early and late stage osteogenic differentiation markers. The composite scaffolds also showed elevated mineralization as compared to the pure POC scaffold. Cell mineralization is a crucial indicator of osteogenesis in vitro. Our result confirms that the inclusion of bioactive glass into POC can stimulate cellular mineralization (Fig. 7).

Observations of cell morphology, ALP activity and gene expression suggest that osteoblastic differentiation may have occurred within the first two weeks of culture on the POC-BG composite scaffolds. Furthermore, this response of hBMSCs on composite scaffolds was independent of stimulation with osteogenic media, indicating that the scaffolds alone can promote the osteogenic responses. We found that hBMSCs grown on the composite scaffolds exhibited superior osteogenic potential (particularly, POC-10%BG) than those found on the pure polymer scaffold. However, this raises the question as to why incorporation of 10%
bioactive glass into the POC might improve the biological response more effectively than higher percentages.

Previous studies have demonstrated that ionic dissolution products released from bioactive glasses can stimulate angiogenesis and osteogenesis [38, 39]. Silicate-based bioactive glasses have been shown to have the ability to support proliferation and differentiation of osteoblastic cells and MSCs either in vitro or in vivo [40, 41]. It has been reported that formation of a calcium phosphate layer on the surface of bioactive glasses can stimulate the adjacent tissues to form new bone in the absence of any osteogenic supplements [42]. Ionic dissolution products released from glasses, particularly Si, play a crucial role in stimulating the proliferation of osteoblast-like cells, up-regulating the expression of a number of osteoblastic genes and promoting the bone growth. Valerio et al. demonstrated that the higher osteoblasts proliferation and collagen synthesis after being treated by bioactive glass dissolution products are related to Si contact but not Ca, because no enhanced osteoblast activity was observed in the absence of Si release [48]. Based on the results discussed above, we believe that the effect of Zn$^{2+}$ and Ga$^{3+}$ on the hBMSCs function can be considered to be more dominant than those of Si since POC-30%BG showed a higher SiO$_2^{2-}$ release [24] but poorer biological response as compared to POC-10%BG. Furthermore, our previous study showed that the concentration of Ga$^{3+}$ and Zn$^{2+}$ in physiological solution increased by enhancing the bioactive glass amount [24]. Therefore, it could be inferred that the presence of a high concentration of the mentioned ions may exert very low cytotoxicity. Even so, the stimulated osteogenic differentiation of hBMSCs observed in the present study is possibly the result of the combined effects of all the ions.

In order to improve the bioactivity of bioactive glasses towards a specific biological response, various metal ions have been incorporated into the glass structure. Studies have investigated the effect of Zn within the bioactive glass composition on the osteogenic differentiation of stem cells [29, 43, 44]. Zinc addition to bioactive glasses has a contradictory effect on cell response, with some studies mentioning the stimulatory effect of Zn on bone formation at up to 5 mol% and others reporting the optimal amount of Zn to be less than 1 mol% [45, 46]. The beneficial effect of Zn is reliant on the dose and duration [47]. Not only Zn compounds stimulate the proliferation of mouse marrow cells but they also inhibit osteoclast-like cell formation [48]. Oh et al. reported that Zn-containing bioactive glasses stimulated the osteogenic differentiation of MSCs as determined by ALP and BSP to an extent equal or even greater than that of Zn-free glass [43]. However, Haimi et al. reported that substitution of CaO with ZnO in a bioactive glass resulted in the suppression of
osteogenic differentiation in the commitment of human adipose stem cell in respect to the Zn-free glass, attributed to the reduction in the degradation profile after Zn addition [46].

Few studies have been conducted to assess the interaction between Ga-containing bioactive glasses and cells [49, 50]. Ga has a beneficial influence on the secretion and synthesis of type I collagen, while it decreases osteocalcin gene expression without affecting on viability of osteoblasts [51]. Furthermore, in vivo implantation of Ga-doped apatitic calcium phosphate cement into a rabbit bone critical defect exhibited osteoinduction without adverse effects [52]. However, this is dose-dependent and is only applicable to up to 14 ppm, which does not induce apoptosis [53, 54]. The results of the current study also confirm that the presence of particular elements in the bioactive glass phase in low concentrations can promote the osteogenic ability of hBMSCs.

This study indicated that composites supported proliferation of hBMSCs less successfully than pure POC, although hBMSCs showed a stronger differentiation capacity when cultured on composite scaffolds. Scaffolds for self-regenerative applications are expected to provide a framework for tissue repair and at the same time act as carriers for antimicrobial agents. The scaffold should be designed to promote cell adhesion, proliferation and differentiation for target tissues whilst also inhibiting bacterial adhesion and biofilm formation. The results of the present study suggest that the POC/bioglass composite support hBMSCs growth and differentiation and can be used as a scaffolding material for bone regeneration in tissue engineering applications. Furthermore, the associated cytotoxicity produced by the release of high concentrations of the ions from bioactive glass phase detected in the confined culture wells may have been modulated in vivo [55, 56].

5. Conclusion

This in vitro study evaluated the response of hBMSCs to composite scaffolds made up of POC and a bioactive glass containing Zn$^{2+}$ and Ga$^{3+}$. The cells adhered, proliferated and differentiated on the scaffolds, even though the morphology, growth, and osteogenic differentiation of the hBMSCs was depended upon the amount of bioactive glass. The cells were flatter on the pure POC than on the composite scaffolds after 14 days of culture. It was found that pure POC had the highest hBMSC proliferation rate, but the weakest effect on osteogenic differentiation. The incorporation of bioactive glass into POC was found to contribute to a solution-mediated effect on osteogenesis of hBMSC. Low levels of bioactive glass content enhanced the osteogenesis of hBMSCs remarkably. The addition of 10%
bioactive glass to POC was proved to significantly promote ALP activity and the mRNA expression of early and late osteogenic markers such as RUNX2, BMP2, COL I, SPARC, and BGLAP, as well as cellular mineralization, suggesting that there is a threshold of bioactive glass content for optimal cellular response. The results demonstrated that composite scaffolds can be used as a potential bone regenerative biomaterial for stem cell based therapies. The strength of most of the polymers and their composite scaffolds are orders of magnitude weaker than natural cancellous and cortical bones. Thus, they could be only used in no-load bearing applications; for example, to obliterate bone cavities resulting from infection or cancer.

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References


Graphical abstract
Highlights

- The responses of mesenchymal stem cells to a poly (octanediol citrate)-bioglass composite scaffold were evaluated in vitro.
- Mesenchymal stem cells appeared to flatten on the pure polymer scaffold while maintaining rounded morphology on the composite scaffolds.
- Composite scaffolds showed enhanced osteogenic differentiation in respect to the unfilled poly (octanediol citrate) scaffold.