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# Incorporation of Human-Platelet-Derived Growth Factor-BB Encapsulated Poly(lactic-co-glycolic acid) Microspheres into 3D CORAGRAF Enhances Osteogenic Differentiation of Mesenchymal Stromal Cells

## Abstract

Tissue engineering aims to generate or facilitate regrowth or healing of damaged tissues by applying a combination of biomaterials, cells, and bioactive signaling molecules. In this regard, growth factors clearly play important roles in regulating cellular fate. However, uncontrolled release of growth factors has been demonstrated to produce severe side effects on the surrounding tissues. In this study, poly(lactic-co-glycolic acid) (PLGA) microspheres (MS) incorporated three-dimensional (3D) CORAGRAF scaffolds were engineered to achieve controlled release of platelet-derived growth factor-BB (PDGF-BB) for the differentiation of stem cells within the 3D polymer network. Fourier transform infrared spectroscopy, energy-dispersive X-ray spectroscopy, scanning electron microscopy, and microtomography were applied to characterize the fabricated scaffolds. In vitro study revealed that the CORAGRAF-PLGA-PDGF-BB scaffold system enhanced the release of PDGF-BB for the regulation of cell behavior. Stromal cell attachment, viability, release of osteogenic differentiation markers such as osteocalcin, and upregulation of osteogenic gene expression exhibited positive response. Overall, the developed scaffold system was noted to support rapid cell expansion and differentiation of stromal cells into osteogenic cells in vitro for bone tissue engineering applications.

## Disciplines

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**Incorporation of Human Platelet Derived Growth Factor-BB encapsulated Poly(lactic-co-glycolic acid) Microspheres into 3D CORAGRAF Enhances Osteogenic Differentiation of Mesenchymal Stromal Cells**

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Short title: 3D CORAGRAF–PLGA microsphere scaffold for controlled PDGF-BB release

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## Abstract

Tissue engineering aims to generate or facilitate regrowth or healing of damaged tissues by applying a combination of biomaterials, cells, and bioactive signaling molecules. In this regard, growth factors clearly play important roles in regulating cellular fate. However, uncontrolled release of growth factors has been demonstrated to produce severe side effects on the surrounding tissues. In this study, poly (lactic-co-glycolic acid) (PLGA) microspheres (MS) incorporated three-dimensional (3D) CORAGRAF scaffolds were engineered to achieve controlled release of platelet-derived growth factor-BB (PDGF-BB) for the differentiation of stem cells within the 3D polymer network. Fourier transform infrared spectroscopy, energy-dispersive X-ray spectroscopy, scanning electron microscopy, and microtomography were applied to characterize the fabricated scaffolds. In vitro study revealed that the CORAGRAF–PLGA–PDGF-BB scaffold system enhanced the release of PDGF-BB for the regulation of cell behavior. Stromal cell attachment, viability, release of osteogenic differentiation markers such as osteocalcin, and upregulation of osteogenic gene expression exhibited positive response. Overall, the developed scaffold system was noted to support rapid cell expansion and differentiation of stromal cells into osteogenic cells in vitro for bone tissue engineering applications.

*Keywords:* CORAGRAF; PLGA; Microsphere; Stromal cell; Platelet-derived growth factor; Osteogenic

## Introduction

Calcium phosphate (CaP) scaffolds composed of beta-tricalcium phosphate,<sup>1</sup> hydroxyapatite, chitosan,<sup>2</sup> and their composites<sup>3</sup> are ideal for bone repair owing to their biocompatibility, adjustable degradation rates, and excellent bioactivity.<sup>4-6</sup> However, they are not osteoinductive in nature, and materials such as chitosan are brittle and exhibit poor cell attachment.<sup>7-9</sup> Although scaffolding materials are critical for tissue regeneration, researchers have found that growth factor stimulation, both in vitro and in vivo, is also vital. Reports have shown that the overall functional bioactivity of scaffolds, including bone or cartilage healing, improved following incorporation of growth factors, such as transforming growth factor-beta (TGF- $\beta$ ), vascular endothelial growth factor (VEGF), and bone morphogenetic proteins (BMPs), into the biomaterial scaffolds.<sup>10</sup> Among the various well-known growth factors, platelet-derived growth factor-BB (PDGF-BB) is a potent mitogen that can induce angiogenesis and direct cell migration, and is involved in vessel maturation and stabilization.<sup>11</sup> In recent times, although increasing numbers of drug-loaded scaffolds have been designed using different structures and materials, the undesirable drug burst release phenomenon associated with rapid drug diffusion from these scaffolds has reduced the effectiveness of the drugs, thus constraining the application of these biocomposites.<sup>12-13</sup> Nevertheless, studies have shown that incorporation of drug-loaded microspheres (MS) into composite scaffolds with gradient structure could effectively solve the problem of drug burst release phenomenon.<sup>14-16</sup> It has been reported that, in addition to scaffolds and growth factors, human primary cells (e.g. mesenchymal stem cells (MSC), osteocytes, and endothelial cells) supplemented with growth factors or cytokines can also be used to repair bone tissues.<sup>17</sup> Growth factors such as BMP2, TGF- $\beta$ , and Wnt ligands affect cellular migration and proliferation as well as osteogenic differentiation of MSC during bone repair.<sup>18-19</sup> In addition, they can regulate the expression of Runt-related transcription factor 2 (Runx2) and osterix (Osx) through intracellular proteins or

transcription factors, including b-catenin, Smad1/5, and Smad2/3.<sup>20</sup> Ideally, the key growth factors can be programmatically encapsulated and embedded into CaP scaffolds, and then released into the microenvironment of the bone graft after being implanted. These growth factors can subsequently stimulate the expression of genes responsible for osteoblastic differentiation from MSC to pre-osteoblasts and active osteoblasts<sup>21</sup> through a variety of signaling pathways.<sup>22</sup>

Coral has been used as a bone substitute for more than a decade. This material makes a good choice for fabricating scaffolds because it can be easily molded into any desired shape and size.<sup>23</sup> Earlier reports have shown that coral-based biomaterials are biocompatible and osteoconductive.<sup>24-25</sup> Besides, coral can be used as a delivery system for the bone growth factors owing to its adhesive property.<sup>26</sup> The coral graft has interconnected porous structures that offer a large surface area for cell attachment. In addition, it also provides a suitable environment for nutrient transfer and physiological support. Coral-based biomaterials, such as Biocoral and Algipore®, have been widely used in the augmentation of periodontal defects and nonunion in pre-clinical and clinical models.<sup>27-28</sup> Poly(lactic-co-glycolic acid) (PLGA) has been extensively employed in the form of microspheres or nanospheres for the controlled delivery of peptides or proteins owing to its excellent biological properties, including biodegradability, biocompatibility, nontoxicity, bacteriostaticity, and strong adhesion.<sup>29</sup> During such encapsulation process the biological activity of the growth factors were much retained as the unwanted degradation of growth factors was prevented. Although, adsorption of growth factors onto the surface of natural or synthetic polymer matrices will allow the local delivery of growth factors this process will lead in compromising the release kinetics.<sup>30-31</sup> For a successful tissue engineering application it's advantageous to develop a construct that contains a carrier for successful delivery of growth factors and a 3D scaffold of cell attachment and differentiation.

Although previous studies have reported on the incorporation of MS into nanofibrous scaffold for the controlled release of growth factors such as PDGF-BB,<sup>32-33</sup> these scaffolds have limited hard-tissue engineering applications owing to their biomechanical properties. Consequently, in the present study, for the first time, we attempted to develop an ideal hard scaffold system based on the hypothesis that culturing of bone marrow stromal cells in coral graft with controlled release of PLGA–PDGF-BB could induce differentiation of bone marrow stromal cells to bone-like cells. **Such simple yet effective approach could pave the way for further developments in the field of implantable scaffolds for bone tissue engineering.**

## **Materials and Methods**

### **Materials**

PLGA (glycolic acid:lactic acid at a ratio of 50:50, Mw=64 kDa) was purchased from DURECT Corporation (Pelham, AL, USA). Recombinant PDGF-BB was obtained from Life Technologies (Invitrogen, USA). Dichloromethane, tetrahydrofuran, hexane, and polyvinyl acid (PVA) were purchased from Sigma Aldrich (USA). All the other chemicals used were of analytical grade.

### **Preparation of Coragraft scaffold**

Coral from the Porites species was processed using lab standardized protocol. Debris was washed out of the coral using distilled water. Coral was cut and processed into blocks of 5.0 mm X 5.0 mm according to the requirements of the study. These cubical were treated chemically followed by freeze drying. Samples were then radiosterilized at Malaysian Nuclear Agency using gamma irradiation.

### **Preparation of PDGF-loaded MS by double emulsion method**

The MS were prepared by using the double emulsion method.<sup>34</sup> The PVA solution was prepared by adding 5 g of PVA to 100 ml of distilled water, and the mixture was heated and mixed using a thermal magnetic stirrer until all the PVA dissolved. Subsequently, the PLGA emulsion was dropped into the PVA solution using a syringe, and the solution was sonicated for 20 s. Then, the solution was left on a magnetic stirrer for 4 h. The MS were collected by centrifugation in a 50-ml centrifuge tube. The collected solid MS were washed with distilled water at least thrice, freeze-dried, and stored at  $-80^{\circ}\text{C}$ .

The main water-in-oil (abbreviated as w/o) emulsion was prepared by emulsifying PDGF-BB (PDGF- BB 25  $\mu\text{g}/\text{ml}$  in a buffer of sodium acetate at  $\text{pH}=6.3$ , specific volume (1 ml) of 10% PLGA (in dichloromethane solution via exploiting a sonicator at 15W (Dakshin, India) for 20s over an ice bath. The prepared w/o emulsion was subsequently mixed with 2 ml of PVA solution (1% in water) by agitation to establish a secondary water-in-oil-in-water (abbreviated as w/o/w) emulsion. Subsequently, the solution was stirred in a magnetic stirrer for 3h at room temperature to evaporate dichloromethane and the resulting solution was centrifuged (2500g, 5 minutes) to yield microspheres. The yielded microspheres carefully rinsed with deionized water multiple times, lyophilized and subsequently stored in vacuumed cabinet.

### **Incorporation of PLGA MS into three-dimensional CORAGRAF**

The PLGA MS with FITC-BSA were incorporated into three-dimensional (3D) CORAGRAF scaffolds, The PLGA MS with FITC-BSA were prepared by using the double emulsion method where 1g of PLGA was dissolved in 10 ml of dichloromethane and emulsified with 100 $\mu\text{l}$  of FITC-BSA solution employing a sonicator at 200 V for 20 s, microspheres were then collected through freeze-dry method. Briefly, 5 mg of dry PLGA MS were suspended in 500  $\mu\text{l}$  of hexane. Subsequently, 80  $\mu\text{l}$  of the suspension was cultured into the block scaffold, and the scaffold was forsaken in room atmosphere for half an hour to allow the evaporation of the



solvent. Then, another 80  $\mu\text{l}$  of the suspension were seeded on the opposite side of the coragraf scaffold. The entire step has been repeated frequently for two times, leading to the incorporation of approximately 5 mg of MS onto the scaffold. After that, the scaffold was treated with a mixed solvent comprising hexane/THF (90/10 v/v) to anchor the MS on the scaffold and lyophilized to get rid of the solvent. As a control, scaffolds with PLGA MS deprived of growth factors were employed. The concentration of the MS precursor and seeding times were adjusted accordingly to control the accumulated amount of MS on the scaffold.

### **Physicochemical characterization of the biocomposite scaffolds**

The surface morphologies of 3D CORAGRAF with and without PLGA MS were studied under a scanning electron microscope (SEM; Model JEOL JSM-6360, Japan), and the elemental composition of the scaffolds was semi-quantitatively investigated using SEM-energy dispersive X-ray spectroscopy (SEM-EDX) (INCA Energy 200, Oxford Inst). To determine the average size of the fabricated MS, the samples were run through Malvern Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., England). Furthermore, microtomography (micro-CT) system (Scanco Medical XtremeCT HRpQCT) was used to quantify the 3D microstructural properties of the scaffolds, and the Brunauer–Emmett–Teller (BET-Autosorb-iQ2) specific surface area of the biocomposites was measured from the nitrogen adsorption–desorption isotherms. To identify the functional groups of the scaffolds, Fourier-transform infrared spectroscopy (FTIR; Bruker, tensor 27; Germany) analysis was performed in a frequency range of 4000–400  $\text{cm}^{-1}$ . The X-ray diffraction (XRD) patterns of the composites were obtained using PANalytical Empyrean XRD (USA) with monochromatic CuK $\alpha$  radiation ( $\lambda = 1.54056 \text{ \AA}$ ), operated at 45 kV, 40 mA, step size of 0.026°, and a scanning rate of 0.1  $\text{s}^{-1}$  over a  $2\theta$  range from 20° to 80°. The glass transition temperature of the PLGA MS was determined using differential scanning calorimeter (DSC, METTLER TOLEDO 820C-

Error  $\pm 0.25-1^{\circ}\text{C}$ ) at a heating rate of  $4^{\circ}\text{C}/\text{min}$ . The weight loss and thermal stability of the scaffolds were ascertained by thermogravimetric analysis (TGA, Mettler Toledo-SDTA851) at a heating rate of  $5^{\circ}\text{C}/\text{min}$  and temperature of  $30^{\circ}\text{C}-800^{\circ}\text{C}$ .

### **Cell morphology**

After incubation for a few days, the samples were removed from the culture plates, washed thrice with PBS, and fixed with 3% glutaraldehyde in PBS for 24 h at  $4^{\circ}\text{C}$ . Subsequently, the samples were thoroughly washed with PBS and sequentially dehydrated twice in 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol for 15 min each. The fixed samples were freeze-dried, sputter-coated with gold, and examined under SEM.

### **Isolation of MSC**

Human bone marrow aspiration from the femoral medullary canal was performed concomitantly during knee arthroplasty at the University Malaya Medical Center (UMMC), Malaysia, with the patient under general anesthesia. The procedure was performed under aseptic conditions by an experienced surgeon. Prior to knee arthroplasty, informed written consent was obtained from each bone marrow donor. During knee arthroplasty, approximately 2 ml of bone marrow sample were aspirated from the patient's femoral medullary canal using a needle and syringe, transferred into a BD vacutainer, and transported to the cell culture lab. The bone marrow samples were collected from the operating room only after completion of the bone marrow aspiration procedure by the surgeon. The bone marrow collection procedure for the present study was approved by the medical ethics committee (Approval ID No. MECID.NO: 201412-859, UMMC). The cell isolation procedure was performed in accordance with the approved guidelines of UMMC.

Subsequently human bone marrow stromal cells were harvested and cultured as previously described.<sup>35</sup>

### **Cell seeding and culture**

The MSC suspension at a density of  $3.0 \times 10^4$  cells/mL was seeded onto the PLGA MS incorporated CORAGRAF. Subsequently, the MSC-seeded scaffolds were left for 28 days in DMEM to induce osteogenic differentiation, and the medium was changed every 4 days.

### **In vitro release of PDGF-BB**

In a 6-well plate, 10  $\mu$ g of the dispersed PLGA MS were incubated in 1 ml of DMEM, and the medium was collected at certain time points. The procedure was simultaneously repeated with PLGA MS embedded onto the CORAGRAF. The media collected were assessed using the PDGF-BB ELISA kit (eBioscience, USA) according to manufacturer's instructions.

### **Confocal microscopy**

Confocal microscopy was performed to determine the cell density on the surface of the scaffolds and infiltration of the cells into the pores of the scaffolds. The MSC-seeded scaffolds were stained with Hoechst 33342 cell-permanent nuclear stain (Life Technologies, Invitrogen, USA). The molecular probes got bound to the A-T regions of the DNA and emitted a blue fluorescence at 460 nm. The morphology and distribution of the MS in the scaffold were examined using SEM and laser scanning confocal microscopy (Leica TCS-SP5 II; Leica Microsystem, Mannheim, Germany). The scaffolds with human bone marrow stromal stem

cells were stained according to the manufacturer's protocol and analyzed using confocal microscopy (Leica TCS-SP5 II; Leica Microsystem, Mannheim, Germany).

### **Alamar Blue assay**

The nontoxic Alamar Blue assay (Invitrogen) was performed to measure the cell proliferation in PLGA MS loaded CORAGRAF on days 0, 3, and 7 post-seeding. In a 96-well plate, 1/10th volume of the AlamarBlue® reagent was added to the cells in the culture medium (cell density =  $1.0 \times 10^4$  cells/mL), and the mixture was incubated in 5% CO<sub>2</sub> at 37°C for 4 h. Subsequently, a 100- $\mu$ l aliquot was placed in a microtiter plate reader (Biotek, Epoch USA) and the absorbance measurements were determined at 570 and 600 nm.

### **Osteocalcin ELISA**

Osteocalcin activity on the scaffolds was determined on days 0, 3, and 7 using the osteocalcin ELISA assay kit (eBioscience). First, the cell culture sample was incubated at room temperature (18 to 25°C) for 2 h, and then, following a few wash procedures according to the manufacturer's protocol, 100 $\mu$ l substrate solution was added and incubated for 10 minutes. Subsequently, the absorbance measurements were determined at 450 nm using a spectrophotometer (Biotek, Epoch USA).

### **Alizarin red staining**

Alizarin red staining was performed to identify the calcium deposits on the scaffolds. A total of 500  $\mu$ l of Alizarin red were added to the cell culture plate and incubated for 20 min. Subsequently, the plate was continuously washed with PBS to remove any additional stain. The culture plate images were captured using (Nikon, Japan) T3 microscope.

## **Gene expression**

To examine the level of osteogenic differentiation, gene expression was employed according to a previously established method.<sup>36</sup>

## **Results**

### **Morphological analysis of the scaffolds**

SEM analysis was performed to assess the patterns of pore diameter and surface of the scaffolds (Fig. 1a–c). While the pure CORAGRAF had a pore diameter of 107–315- $\mu\text{m}$ , the pores of PLGA MS loaded CORAGRAF were partially infiltrated with MS and a few pores remained open. Analysis of the PLGA MS using Mastersizer (Fig. 1d) indicated that the average particle size to volume % was around 137  $\mu\text{m}$ . Furthermore, confocal images confirmed (Fig. 2a–c) that the PDGF-BB-loaded PLGA MS infiltrated up to 599- $\mu\text{m}$  thickness of the 3D scaffold material. The PLGA MS loaded with FITC-BSA are shown in Fig. 2c. The 3D image obtained by incorporating multiple series of images collected by confocal laser microscopy further aided the investigation of PLGA infiltration.

### **Porosity analysis**

The 3D CORAGRAF scaffolds with and without PLGA MS were subjected to micro-CT examination (Fig. 3a, b). The total volume of the 3D CORAGRAF without PLGA MS was 432.81  $\text{mm}^3$ . The material volume of the CORAGRAF without PLGA MS was 169.22  $\text{mm}^3$ , while that of the CORAGRAF with the PLGA MS was 288.58  $\text{mm}^3$ . The ratio of the total volume and material volume for the CORAGRAF without and with PLGA MS was about 0.39 and 0.66, respectively. These results clearly indicated that almost 50% of the CORAGRAF was infiltrated with PDGF-BB-loaded PLGA MS. The porosity of the CORAGRAF without the PLGA MS was about 60%; however, after PLGA MS incorporation, the porosity decreased to

about 33%. Furthermore, micro-CT was also employed to measure the material surface area of the CORAGRAF without and with MS, which was 2031.12 and 2199.01mm<sup>2</sup>, respectively.

Comparative BET analysis (Fig. 4a, b) of the scaffold materials revealed that both the scaffolds possessed a typical type IV isotherm with a well-defined presence of mesopores. However, the pore-size distribution calculated by Barret–Joyner–Halenda (BJH) method revealed that the total pore volume of the mesopores was considerably lower in CORAGRAF with PLGA MS, when compared with that in CORAGRAF without PLGA MS, which could be owing to the high total volume of the macropores assigned to the PLGA MS. In addition, CORAGRAF without PLGA MS showed a significantly higher specific surface area (32.67 m<sup>2</sup>/g) and specific pore volume (0.019 cm<sup>3</sup>/g), when compared with CORAGRAF with PLGA MS (2.36 m<sup>2</sup>/g and 0.0063 cm<sup>3</sup>/g, respectively), which might be owing to the space occupied by the PLGA MS.

### **Characterization of the scaffolds**

FTIR spectroscopy was employed to study the functional groups of the scaffolds, and the results are shown in Fig. 5a. Both the CORAGRAF scaffolds with and without PLGA MS showed the characteristic peaks of carbonate, including the absorption bands of 706, 853, 1081, and 1447 cm<sup>-1</sup> corresponding to in-plane bending, out-of-plane bending, symmetric stretching, and asymmetric stretching vibrations of the carbonate ions, respectively.<sup>37-40</sup> However, CORAGRAF with PLGA MS revealed four new absorption peaks (which were not observed in CORAGRAF without PLGA MS) at 1176, 1265, 1753, and 2921 cm<sup>-1</sup> corresponding to C-O stretching, CH<sub>2</sub> and CH<sub>3</sub> asymmetric angular deformation, C=O stretching, and C-H stretching vibrations, respectively,<sup>41-46</sup> which proved the presence of PLGA in this biocomposite.

The polymorphism of the scaffolds was examined by XRD, and the XRD patterns of both the scaffolds are shown in Fig. 5b. The XRD spectra of the two scaffolds revealed sharp

peaks (indicated by \* in Fig. 5b) corresponding to the crystalline planes of aragonite (JCPDS card No. 41-1475). However, no peak of vaterite or calcite was noted, indicating that the CORAGRAF was nearly pure aragonite.<sup>47</sup> It should be noted that the encapsulated PLGA MS did not show any peak in the XRD spectrum, suggesting that the addition of MS did not change the CORAGRAF polymorphism and that the PLGA particles were amorphous in nature.<sup>48-49</sup> Furthermore, to confirm the types of elements present in the CORAGRAF, EDS was employed, and the results revealed the presence of moderate carbon levels and high carbon and calcium levels (Fig. 5c). Moreover, the levels of these elements were not significantly altered after the incorporation of PLGA MS.

### **DSC and TGA analyses**

DSC and TGA analyses were conducted to further investigate the existence of PLGA MS in the CORAGRAF, and the results are illustrated in Fig. 6a and 6b, respectively. While the DSC thermogram of the CORAGRAF without PLGA MS did not show any peak in the designated temperature range, that of the CORAGRAF with PLGA MS presented an endothermic peak at 51°C, corresponding to glass transition temperature ( $T_g$ ) of PLGA.<sup>50</sup> As indicated by the TGA curve of pure CORAGRAF (Fig. 6b), the  $\text{CaCO}_3$  crystals revealed two main steps of weight loss. The first step of weight loss was observed at around 300°C, exhibiting about 1% decrease in weight, which can be attributed to evolution of  $\text{CO}_2$  from  $\text{CaCO}_3$ .<sup>51</sup> The second step of weight loss was noted with a higher slope at 575°C–780°C, with a weight loss of about ~42%, which indicated decomposition of  $\text{CaCO}_3$  to  $\text{CaO}$ .<sup>52-53</sup> However, with the addition of PLGA MS, the weight-loss pattern exhibited significant difference at 100°C–600°C, with a considerably large amount (6%) of mass loss in CORAGRAF with PLGA MS, when compared with that in pure CORAGRAF. In particular, the weight loss at 100°C–240°C can be attributed to evaporation of the solvent, while that at 240°C–600°C can be related to decomposition of

PLGA, with major decomposition corresponding to combustion of organic compound occurring at 300°C (which overlapped with conversion of CaCO<sub>3</sub> to CO<sub>2</sub>). Thus, it can be concluded that the loading capacity of PLGA MS on the CORAGRAF was about 6%.

### **Cell viability and bone markers expression**

Alamar Blue assay was performed to confirm cell viability throughout the experimental period (Fig. 7b). The cell viability gradually increased, which was indicated by gradual dye reduction. When compared with early time points (days 0 and 3), the latter time points presented significant increase in cell viability ( $p < 0.05$ ). Moreover, confocal microscopy results confirmed the DNA staining of the cells inside the coral graft (Fig. 7a).

As shown in Fig. 8a, considerably intense alizarin staining was evident in the bone marrow stromal cell cultures treated with PLGA MS containing PDGF-BB, whereas no positive staining was observed in the untreated cultures. Moreover, addition of PDGF-BB alone to the cultures also showed positive alizarin staining, although the intensity of staining (red dye) was less. These results suggested that the controlled release of PDGF-BB from the PLGA MS incorporated onto the CORAGRAF scaffold may be beneficial for rapid cell expansion and differentiation of stromal cells in 3D environment.

The controlled release of PDGF-BB was monitored using two different groups at variable time points (Fig. 8b). Incubation of PLGA MS encapsulated with PDGF-BB in the medium showed an initial burst and relatively high PDGF-BB release on days 0 and 3, and a subsequent reduction in the release of the growth factor on day 7. In contrast, the PDGF-BB-encapsulated PLGA MS incorporated onto the 3D CORAGRAF presented a relatively low release of approximately 200 pg/ml PDGF-BB on days 0, 3, and 7, and the quantity was almost the same at all the three time points. These results indicated that the release of PDGF-BB from the PLGA MS incorporated 3D scaffold was well controlled. Furthermore, the cells treated



with PLGA MS containing PDGF-BB showed a twofold increase in the release of osteocalcin on day 7 (Fig. 8c), when compared with that on day 0. In contrast, the cells treated with PDGF-BB did not show any increase in osteocalcin release. Moreover, monitoring of the release of osteocalcin from the PDGF-BB-PLGA MS-CORAGRAF scaffold at various time points clearly indicated that the release of osteocalcin gradually increased, and was significant on day 21, when compared with that on earlier time points (Fig. 8d).

Subsequently, we examined the expression of an array of genes related to osteogenesis, including Runx2, osteopontin (OPN) (Fig. 9a, b), collagen1, BMP2 (Fig. 10a, b), bone gamma-carboxyglutamate (Gla) protein (BGALP), and osteonectin (ON) (Fig. 11a, b) at different time points (days 0, 7, 14, and 21). On day 14, the expression of Runx2 and OPN was significantly high, exhibiting approximately three- and twofold increase, respectively, in the PDGF-BB-loaded PLGA MS-CORAGRAF scaffold, when compared with that in the CORAGRAF scaffolds without PLGA MS and PDGF-BB. In addition, the expressions of collagen1 and BMP2 were significantly increased ( $p < 0.05$ ) in the PDGF-BB-loaded PLGA MS-CORAGRAF scaffold, when compared with those in the CORAGRAF scaffolds without PLGA MS and PDGF-BB; however, a decline in the expressions of these genes was noted on day 21. Similarly, the levels of BGALP and ON were also significantly higher ( $p < 0.05$ ) on day 14 in the PDGF-BB-loaded PLGA MS-CORAGRAF scaffold, when compared with those in the CORAGRAF scaffolds without PLGA MS and PDGF-BB.

## Discussion

The use of CORAGRAF in tissue engineering has gained potential interest owing to its osteoconductive property and compatibility with the in vivo models.<sup>54</sup> In the present study, to improve its osteogenic differentiation property, we incorporated PLGA MS containing PDGF-BB into the CORAGRAF scaffold for the controlled release of PDGF-BB. The SEM and Mastersizer data indicated that the scaffold environment could support the growth and differentiation of stromal cells. PLGA microspheres can be prepared using polymers of different molecular weight that can influence the release profiles of target proteins. Herein, we were capable of attaining the sustained release of PDGF-BB from PLGA50 microspheres implanted in the scaffolds. Microsphere encapsulation of growth factors protects them from degradation and deactivation when compared to direct supplementation of growth factors. According to our in vitro bioactivity assay PDGF-BB has retained its bioactivity during loading into PLGA microspheres. Loading of growth factors into the microspheres and subsequent incorporation of such cargo into 3D scaffolds serves as a golden gate toward yielding a controlled release profile for growth factors as well as sustaining their bioactivity for a longer duration of time. Of note, the mechanism behind such findings is yet to be discovered.<sup>55</sup> In clinical applications for the treatment of various bone ailments, the general biology of the bone marrow stromal cells is the focus of interest.<sup>56</sup> In the present study, although only a few pores of the CORAGRAF were found to be infiltrated by PLGA MS, the CORAGRAF provided a conducive environment for cell attachment on its surface. The cells attached onto the CORAGRAF scaffold were randomly distributed within the pores and on the MS with an average size of approximately 137  $\mu\text{m}$ . Furthermore, the attached cells showed roughened blebs and protrusions on the scaffold surface as well as a fibroblast-like appearance in some regions. It has been previously reported that for achieving osteoblast-like behavior of the cells, factors such as cell attachment efficiency and dissemination rely on the surface chemistry of

the material,<sup>57</sup> which was found to be positive in the present study. Furthermore, cell infiltration into the PLGA MS incorporated CORAGRAF was random.

Previous studies have shown that coral is an excellent material for bone repair. Coral grafting of osteochondral defects has been reported to produce new bone with characteristics similar to those of normal subchondral bone in rabbits. Some pre-clinical reports and clinical trials have confirmed that coral has good biocompatibility properties, and there have been no reports on any rejection or collateral effects of coral. Moreover, it has been demonstrated that coral can be used as an osteoconductor.<sup>58</sup> However, some recent studies have proved that stromal cell proliferation without growth factors in 3D scaffolds presents important limitations. Consequently, many studies have used expensive commercial osteogenic media for cell differentiation protocols.

In the present study, to achieve a cell confluence in a few days, similar to that obtained in conventional culture plates, we employed the controlled growth factor release strategy for releasing PDGF-BB from PLGA MS incorporated CORAGRAF scaffolds. PDGF-BB stimulates the proliferation and recruitment of both periodontal ligament and bone cells *in vitro*. Some *in vivo* studies have demonstrated that this growth factor enhances periodontal regeneration in beagle dogs and non-human primates, heals bone defects in humans, and enhances tissue regeneration, wound healing, and foot ulcers.<sup>59</sup> However, despite these advantages, to maintain adequate therapeutic effect, PDGF-BB needs to be injected in high dose or injected repeatedly.<sup>60</sup> This is not practically applicable in most cases because of the high costs and multiple administrations, which may necessitate additional surgical procedures *in vivo*. Based on previous studies, we presumed that a slower release of PDGF-BB would keep the growth factor intact and functionally active when released into the culture. In this regard, we examined different strategies *in vitro*. It must be noted that alizarin staining of PDGF-BB-encapsulated PLGA MS–CORAGRAF was too intense because of the presence of CaCO<sub>3</sub> in

CORAGRAF. Hence, the difference in staining between the 3D CORAGRAF with and without PDGF-BB-encapsulated PLGA MS could not be assessed at the early stage of differentiation.

To the best of our knowledge, the present study is the first to report on rapid cell expansion and osteogenic differentiation using CORAGRAF with PDGF-BB-encapsulated PLGA MS. The morphological changes that occurred in the developed biocomposite provided a suitable environment for the cells to get attached onto the surface. Although some decline in the porosity was observed, these changes were inevitable owing to MS incorporation, which favored greater surface area without affecting cell viability. Besides, scaffolds provide a conducive environment for the growth of multipotent bone marrow derived stromal cells and stimulate their osteoconductive and osteoinductive properties that contribute to bone repair. Previous studies have shown that bone marrow stromal cells isolated from the marrow of mice, rats, rabbits, dogs, and humans can be expanded in vitro. These cells can be differentiated into osteoblast-like cells under certain conditions using commercial osteogenic media and multiple growth factor supplements such as BMP.<sup>61</sup>

While PDGF-BB can induce proliferation of stromal cells and osteogenesis,<sup>62</sup> there are conflicting reports on the ability of PDGF-BB to inhibit osteogenic differentiation of osteoclast cell lines in conventional culture flasks.<sup>63</sup> It has been reported that markers of mature osteoblasts such as alkaline phosphatase (ALP), osteocalcin, and type I collagen were inhibited in the pre-osteocytic cell lines.<sup>64</sup> However, some studies have demonstrated that PDGF-BB does not alter the marker activity and mineralization in human stromal cells. It has been indicated that imatinib-induced blockade of PDGFR- $\beta$  produced opposing effects on the differentiation of bone marrow stromal cells.<sup>65</sup> Another study revealed that PDGF-BB with peptides could improve the proliferation, differentiation, and early calcification of osteoblast cells.<sup>66</sup> Among the many major growth factors, PDGF-BB is the only growth factor currently approved by the US-FDA for periodontal regeneration as a part of dental bone filling device.

Furthermore, PDGF-BB has been incorporated into the MS in nanofibrous materials for osteogenic response by slow delivery of the growth factor. Our previous studies have demonstrated that some nanofibrous material and fucoidan-based scaffolds promoted differentiation of bone marrow stromal cells with PDGF-BB supplementation.<sup>67-68</sup> In the present study, the release of the osteogenic differentiation marker, osteocalcin, confirmed that the controlled release of PDGF-BB from the 3D scaffold is beneficial for osteogenic differentiation. Despite the proven efficacy of PDGF-BB, its biomedical application is complicated by its short biological half-life, systemic side effects, rapid clearance, and lack of biomechanical property of the fibrous materials. Therefore, delivery systems that maximize the therapeutic abilities of PDGF-BB are desirable, not only for enhancing bone formation, but also for limiting undesirable biological reactions. In previous studies, to retain PDGF-BB at the desired site for a prolonged time period, delivery via PLGA MS has been employed.<sup>36</sup>

Intramembranous ossification has been known as the usual mechanism deployed by the biomaterials to induce bone formation; yet, endowing growth factors induces bone formation via endochondral ossification. In spite of the fact that growth factors showed efficacy in inducing osteogenesis, materials with osteoconductive traits are preferred for preclinical studies. Nonetheless so far no studies have been devoted to comparing the performance of biomimetic composites against growth factor supplementation in inducing osteogenesis. This could be due to variety of the approaches employed for the purpose of fabricating a biomimetic composite scaffold (either in nanoscale or microscale) and the choice of cell line, i.e. primary or immortal cells. In fact some of the most crucial phenomenon involved in cell development cascade such as cell contraction, migration, and intracellular signaling are entitled to improvement as a consequence of enhanced cell attachment. Overall, in comparison to two-dimensional surface, 3D surface offers extra dimension which subsequently enhances the interactions

between cell and the matrix as well as communications among cells, that were known vital to the physiology of the cells.<sup>69</sup>

In the present study, the considerable increase ( $p < 0.05$ ) in the expression of Runx2, the central control gene within the osteoblast phenotype, on day 14 indicated the ability of the developed scaffold system to support osteogenic differentiation. A previous work had demonstrated that recombinant PDGF-BB can increase the activity of Runx2 as well as regulate the downstream genes that maintain osteoblastic phenotype such as ALP. Moreover, while the gene expression in human MSC seeded onto collagen nanofibers has been reported to mitigate after passing of 3 weeks<sup>70</sup>, in this study, the decline was yielded after day 14 in the developed 3D scaffold. These findings showed the short window between start and ending of the differentiation phenomenon of human MSC grown on CORAGRAF with PDGF-BB-encapsulated PLGA MS. Collagen is among crucial proteins expressed all through bone differentiation. Herein, collagen gene expression was apparent in the early time point, underscoring the primary cellular response to osteoinductive factors. Furthermore, in accordance with previous reports, collagen was considerably expressed after day 7, and was extracellularly produced leading to formation of an well integrated matrix within the scaffold structure. This is a crucial step due to imperative role of collagen in stimulates pre-osteoblast cell surface integrin that leads to activation of other core binding factors, which is known as an essential part of osteogenesis.<sup>70</sup> The expression of OPN (as a function of time) during the differentiation phase (in presence or absent of extracellular molecules) followed the same pattern as ON expression. Previous reports have demonstrated that the level of OPN is high during the differentiation period.<sup>71</sup> In addition, a previous study revealed that the OPN expression escalated at the inauguration of osteodifferentiation and subsequently reduced during 21 days of differentiation. In the same manner, another study reported a decline in OPN expression in human MSC after 1 week of differentiation.

The increase in BMP2 noted on day 14 in the present study primarily showed that the developed scaffold could initiate particular differentiation of stem cells or progenitors to form osteoblast-like cells. PDGF-BB-induced nodule formation was increased in the presence of BMP2, indicating the strong probability of signaling between PDGF-BB and BMP2 pathways. Moreover, PDGF-BB requires the presence of BMPs for the induction of bone formation, suggesting that BMP signaling could be involved in PDGF-BB-based stem cell gene therapy. In addition, this may also explain the ability of PDGF-BB to enhance bone formation in bone but not in other tissues. It has been reported that the increased expression of some osteogenic markers such as OPN and osteocalcin (BGALP) ( $p < 0.05$ ) indicates early osteogenic differentiation phase was perfectly funded by 3D environment.<sup>72-73</sup> Osteocalcin is predominantly expressed by the osteoblastic phenotype, and is a reliable marker in osteogenic differentiation and subsequent mineralization.<sup>74</sup> In connection with the BGALP quantification data, the post-proliferative osteoblast maker, osteocalcin, was also used in the present study to assess the differentiation of human MSC on CORAGRAF with PDGF-BB-encapsulated PLGA MS. The osteocalcin gene expression was observed at an early time point, which could be a consequence of the partial differentiation of human MSC on the scaffold. This finding was corresponding to the alizarin red staining results, which revealed that the staining was inhomogeneous throughout the sample.

In conclusion, the developed PDGF-BB-encapsulated PLGA MS-CORAGRAF scaffold system was capable of releasing PDGF-BB in a controlled manner for a prolonged duration with significant reduction in burst effect. The stromal cell attachment, viability, release of osteogenic differentiation markers such as osteocalcin, and upregulation of osteogenic gene expression were considerably positive. The findings of this study could aid in the development of a strategy to construct a powerful delivery vehicle to overcome a well-described challenge in tissue engineering.

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## Conflict of interests

The author(s) declare no competing financial interests.

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### **Author contribution**

SM, PK, EN, and TS executed the lab work, HBR, MRM, KG designed the work protocol, constructed the graphs and prepared the manuscript. TK edited the manuscript.

### **Figure legends**

Figure 1a, b and c. Scanning electron microscopy of CORAGRAF (1a), The PLGA microsphere encapsulated into the CORAGRAF (1b), CORAGRAF with PLGA microsphere with human bone marrow stromal cells (1c). All the samples were fixed with 3% glutaraldehyde in PBS for 24 h at 4 °C and processed. Figure 1d Mastersizer of PLGA

microsphere using Malvern Mastersizer 2000 particle size analyzer showing the average particle size of 137  $\mu\text{m}$ .

Figure 2a-c. Infiltration of PLGA microsphere in CORAGRAF culture system using the confocal microscopy. Green color indicates regions stained with FITC in the range of approximately 599  $\mu\text{m}$  of 3D CORAGRAF.

Figure 3 a & b Micro-ct analysis of CORAGRAF and CORAGRAF-PLGA loaded scaffold. A Micro-CT system (Scanco medical XtremeCT HR pQCT) was used to quantify the 3D microstructural properties

Figure 4 a & b BET of CORAGRAF (a) and PLGA microsphere encapsulated into CORAGRAF (b). The Brunauer–Emmett–Teller (BET-Autosorb-iQ2) specific surface area of the biocomposites was measured from the nitrogen adsorption–desorption isotherms. *Inset* is the corresponding BJH pore size distribution.

Figure 5a-c. Fourier transmission infrared (FTIR) of biocomposites (a), XRD patterns of scaffolds (b) and Energy Dispersive Spectroscopy of CORAGRAF and CORAGRAF PLGA microsphere (c).

Figure 6a-b. DSC and TGA assay along with in-vitro degradation result of bio-scaffold. DSC analysis was performed to confirm the state of PLGA microspheres in CORAGRAF (a), TGA analysis was performed to confirm the existence of PLGA microspheres in CORAGRAF and measure their corresponding weight ratio (b), Degradation of CORAGRAF-PLGA composite scaffolds upon immersion in PBS for 10 days duration.

Figure 7a &b. Viability of the cells in the CORAGRAF- PLGA microsphere culture system. Post-seeded cells on day 7 were fixed and viewed under the confocal microscope after staining the scaffold with Hoechst 33342 cell-permanent nuclear stain. Blue dots indicate the DNA-stained live cells (a), Viability of cell in CORAGRAF-PLGA microsphere loaded bone marrow stromal cells at variable time points. \* $P < 0.05$  indicates the levels of significance (b).

Figure 8a Alizarin staining of the control cells and cells treated with the PDGF-BB released from the microsphere and direct PDGF-BB treatment. 8b Release of the PDGF-BB from the microsphere incubated in the media and CORAGRAF–microsphere culture system incubated in the media. Figure 8c Release of the osteogenic marker osteocalcin at variable time points. Figure 8d Release of osteocalcin in CORAGRAF-PLGA at variable time points. \* $P < 0.05$  indicates the level of significance of marker release.

Figure 9a, b. Quantitative expression of Runx2, osteopontin (OPN) in CORAGRAF, CORAGRAF + PPDGF-BB and CORAGRAF+PLGA+PDGF-BB scaffolds during cell differentiation. The total RNA was extracted from hMSCs cultured on the substrates ( $n = 6$ ) at day 0, 7, 14 and 21 using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Following the cDNA synthesis and qPCR, the relative gene expression was normalized to the GAPDH and baseline expression. The data were represented as the means  $\pm$  Standard deviation (SD). The statistical significant was set at level  $P < 0.05$ , \* Represents the comparison between CORAGRAF, CORAGRAF + PPDGF-BB and CORAGRAF+PLGA+PDGF-BB

Figure 10a, b. Quantitative expression of collagen 1 (Col1) and Bone morphogenic protein (BMP2) in CORAGRAF, CORAGRAF + PPDGF-BB and CORAGRAF+PLGA+PDGF-BB scaffolds during cell differentiation. The total RNA was extracted from hMSCs cultured on the substrates (n = 6) at day 0, 7, 14 and 21 using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Following the cDNA synthesis and qPCR, the relative gene expression was normalized to the GAPDH and baseline expression. The data were represented as the means  $\pm$  Standard deviation (SD). The statistical significant was set at level  $P < 0.05$ , \* Represents the comparison between CORAGRAF, CORAGRAF + PPDGF-BB and CORAGRAF+PLGA+PDGF-BB

Figure 11a, b. Quantitative expression of BGALP and osteonectin (ON )in CORAGRAF, CORAGRAF + PPDGF-BB and CORAGRAF+PLGA+PDGF-BB scaffolds during cell differentiation. The total RNA was extracted from hMSCs cultured on the substrates (n = 6) at day 0, 7, 14 and 21 using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Following the cDNA synthesis and qPCR, the relative gene expression was normalized to the GAPDH and baseline expression. The data were represented as the means  $\pm$  Standard deviation (SD). The statistical significant was set at level  $P < 0.05$ , \* Represents the comparison between CORAGRAF, CORAGRAF + PPDGF-BB and CORAGRAF+PLGA+PDGF-BB