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Modified poly(caprolactone trifumarate) with embedded gelatin microparticles as a functional scaffold for bone tissue engineering

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ABSTRACT: Bone tissue engineering offers high hopes in reconstructing bone defects that result from trauma, infection, tumors, and other conditions. However, there remains a need for novel scaffold materials that can effectively stimulate ossification with appropriate functional properties. Therefore, a novel injectable, biodegradable, and biocompatible scaffold made by incorporating modified poly(caprolactone trifumarate) (PCLTF) with embedded gelatin microparticles (GMPs) as porogen is developed. Specifically, *in vitro* and *in vivo* tests were carried out. For the latter, to determine the osteogenic ability of PCLTF-GMPs scaffolds, and to characterize bone-formation, these scaffolds were implanted into critical-sized defects of New Zealand white rabbit craniums. Field Emission Scanning Electron Microscope (FESEM) demonstrated cells of varying shapes attached to the scaffold surface *in vitro*. The PCLTF-GMPs demonstrated improved biocompatibility *in vivo*. Polyfluorochrome tracers detected bone growth occurring in the PCLTF-GMPs filled defects. By incorporating PCLTF with GMPs, we have fabricated a promising self-crosslinkable biocompatible and osteoconducive scaffold for bone tissue engineering. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43711.

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INTRODUCTION

Growing relevant cells into a three-dimensional (3D) tissue construct is one of the principal methods in tissue engineering.[1] To regenerate bone, multidimensional growth of osteoblasts is achieved by making use an osteoconductive porous scaffold to serve as a temporary extracellular matrix that support new bone formation in three dimensions. Hence, the development of a biocompatible material to act as such scaffold and possibly as a carrier for growth factors required for bone regeneration, is the researchers' ultimate aim. In addition, there is also a need to develop *in situ* crosslinkable scaffold materials that are easily implanted or injected into irregularly shaped osseous defects.

Most bone substitutes or scaffolds with osteoconductive properties are available in different forms such as block, granules, powder, and injectable paste. The injectable form is the most preferred because it is easy to work with and allows changes into different shapes. However to crosslink *in situ* (cure), the present injectable material need to undergo either photo-initiation or heating during application. Examples of these products are the resorbable poly(caprolactone) fumarate (PCLF)[2] or the non-resorbable polymethyl methacrylate (PMMA) and Cortoss (resins and reinforcing glass ceramic particles).[3,4] These process, unfortunately could affect the surrounding tissues. Other types of injectable material are calcium and phosphate paste, which hardens to form a ceramic mass within hours. In spite of its recognition as a scaffold that aid bone healing, calcium and phosphate paste suffers from several disadvantages, namely poor injectibility and an inherent lack of microporosity. This means that tissue in growth is impeded, and the material remains inert for a long period with insubstantial resorption.[4,5] One injectable material that has the potential to overcome these issues is poly(caprolactone) (PCL).

PCL is a biodegradable polyester that has been widely used in pharmaceutics and tissue engineering due to its biocompatibility and ability to degrade naturally. However, its strong hydrophobicity, long degradation time and the presence of non-natural cell recognition sites greatly restrict its application in tissue

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engineering.[6] Thus, many kinds of physicochemical and surface modification techniques have been employed to overcome these shortcomings and to improve its degradation rate. PCL can be modified by crosslinking it with a functional group such as the fumarate, to improve its hydrophilicity and the rate of degradation.[7] In the past decade, a resorbable polymeric solution based on 900 Mw poly(caprolactone)-triol (PCL) and fumaryl chloride (FCl), named as the dark poly(caprolactone trifumarate) (PCLTF) and the white poly(caprolactone trifumarate) have sequentially been synthesized.[8,9] Both products are unsaturated, and are made up of highly branched polyesters containing double bonds that originate from the integration of FCl into the poly(caprolactone) backbone. The only difference between them results from the use of different catalysts during esterification i.e., the use of triethylamine results in an opaque dark color PCLTF, while the use potassium carbonate (K_2CO_3) yields a translucent white color in the final product.

The additional non-reacted hydroxyl group (–OH) in the PCL-triol enhances their hydrophilicity, thereby resulting in them to having a higher degradation rate than the original PCL.[8,9] Both the dark and white PCLTF is available as a viscous liquid form that crosslinks *in situ* at room temperature, hence they do not need to be pre-heated before application. This allows for direct application as a bone-filling scaffold, fitting into irregular bony defects with minimal invasive manipulation.

These two biomaterials were produced based on a traditional scaffold fabrication technique namely, the salt leaching method, to create a 3D porous structure. However, there are many issues associated with the use of a high concentration of salt porogen. Some of these salt particles might remain in the scaffold even after the removal of salt porogen, and this itself may cause cytotoxicity to the surrounding tissue. In addition, salt retention may also result in high osmolarity of the surrounding tissue, which leads to local tissue necrosis.[10] Other shortcomings of the previously developed dark and white PCLTF were their availability as a highly viscous polymeric solution (therefore, less flowable) that resulted in decreased injectability, difficulty to produce (this includes handling and the reproducing procedures), as well as the presence of a non-uniform distribution of porogen in the scaffold.[11]

To overcome these shortcomings, a modified form of those pre-existing PCLTFs, now named as the poly(caprolactone trifumarate)-gelatin microparticles (PCLTF-GMPs) scaffold was developed. It was created by mixing 300 Mw PCL with gelatin microparticles (GMPs) which acted as porogen to create a new 3D porous scaffold, as an alternative to mixing the conventional 900 Mw PCL with sodium chloride salt (NaCl) porogen, as previously used in the creation of the white and dark PCLTFs.[11] Gelatin (Gel) is a natural biopolymer, obtained by breaking up the triple-helix structure of collagen into single-strain molecules. It is biodegradable, biocompatible, and non-immunogenic.[12,13] It is well known that gelatin also has many integrin binding sites that attract cellular adhesion and differentiation.[14] Hence, it promotes cell infiltration and proliferation.[15] In addition, it has been reported to significantly enhance the hydrophilicity of the material associated with it, as reported in relation to its presence on the multilayered poly(propylene carbonate) (PPC). As a result, the deposition of gelatin bilayers on PPC remarkably promoted both fibroblast and Human Osteoblasts (HOBs) cell attachment, spread, and growth.[16] This observation was in agreement with other studies that showed favorable chondrocyte and endothelial cells attachment and growth on a modified surface deposited with gelatin and chitosan.[17,18]

The aims of the present study were to improve on a previous form of PCLTF polymer by using gelatin rather than salt as porogen, and to evaluate the cytocompatibility of this PCLTF-GMPs (resultant product) during its different life span *in vitro* i.e., during and after cross-linking, via direct and indirect contact tests. Furthermore, the biocompatibility and morphological feature of the new bone that formed within this modified white PCLTF-GMPs scaffold was assessed *in vivo* using an animal model. The significant difference of this modified material in comparison to the original PCLTF in cranial critical size defects in rabbits was studied.

In this study, by combining the poly(caprolactone trifumarate) (PCLTF) with gelatin microparticles (GMPs), we prepared an injectable, self-crosslinkable, and biocompatible material. This method had greatly improved the original PCLTF containing salt as porogen. Cells treated with the products of the PCLTF-GMPs scaffold showed evidences of growth, with intact cell morphology and nucleus. The PCLTF-GMPs demonstrated improved biocompatibility *in vivo*, while the original PCLTF containing salt was associated with local inflammation and necrosis at the site of implantation. Polyfluorochrome tracers detected bone growth occurring in the PCLTF-GMPs filled defects with the mean values being significantly higher than the control defects. By incorporating PCLTF with GMPs, we have fabricated a promising biocompatible and osteoconducive scaffold for bone tissue engineering.

EXPERIMENTAL

Ethics Statement

For *in vitro* test, the study received approval from the Medical Ethics Committee/IRB, Faculty of Dentistry, University of Malaya [DF OS1211/0071(P)]. Human gingival fibroblasts were harvested from marginal gingival tissue of extracted teeth, obtained during routine orthodontic dental extractions. Since the IRB requirement of the local institution states that tissue of patients that are used for research purpose needs no written consent if there is no requirement to identify the subject, verbal informed consents were taken from the patients prior to storage of the tissue, following the protocol as approved by the ethics committees/IRBs. The participants' approval was written (noted) into the treatment sheet as per their agreement to donate the removed tooth/teeth for the purpose of research. For *in vivo* study, ethical approval was received from the Committee on Animals and Research (2013-07-15/OMS/R/NWC) of the Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC), University of Malaya. All animals were examined by a veterinarian and their suitability for inclusion in this study was confirmed.

Samples Preparation

A white PCLTF-GMPs was synthesized by adopting the method described by Muhammad *et al.*[9] In brief, the modification of the polymer was done using 300 Mw of PCL-triol, and following a molar ratio of 0.9:1:1.2 for the fumaryl chloride (FCl), PCL-triol, and K_2CO_3 (potassium carbonate), and with the use of gelatin microparticles as porogen to create a 3D scaffold. The polymerization process of the PCL-triol, FCL, and K_2CO_3 mixture was maintained at room temperature in a nitrogen atmosphere for 16 h to form a white PCLTF polymer solution. The GMPs porogen was prepared by following a previously established protocol.[10] In brief, 10% gelatin solution was added drop wise into two mineral oils, namely sorbitan monooleate and polyoxyethylene sorbitan monooleate, while stirring at 360 rpm to obtain gelatin microparticles (GMPs) that then crosslinked and were finally collected by filtration.

A PCLTF-GMPs porous scaffold was fabricated by mixing homogenously the PCLTF polymer solution with GMPs at 1:1 ratio. Benzoyl peroxide (BP) 0.0125 g dissolved in 0.175 mL of *n*-vinyl pyrrolidone (NVP) was used as an initiator, and 10 μL dimethyl toluidine solution (DMT) was used as an accelerator. After thorough mixing, the resultant paste was injected into a cylindrical Teflon mold (5 mm in diameter × 1 mm in height) and compressed using a glass plate to create disks with even thickness. The mixture was crosslinked *in situ* and left to solidify within ~ 5–7 min at room temperature. To generate a porous PCLTF structure, the GMPs porogen was leached out by incubating it in 0.1 *N* NaOH solution overnight.

Characterization of Polymers by Fourier Transform Infrared (FTIR) Spectroscopy and Nuclear Magnetic Resonance (^{1}H-NMR) Spectrum

Spectroscopy was used to confirm the formation of the PCLTF polymer solution by identifying the double-bond groups present in the PCL backbones. FTIR (OMNIC 8.1 program, Nicolet 6700, Thermo Scientifics, Madisson, WI, USA) was employed using internal reflection element (IRE) made of diamond as the sample holder. The spectra of the modified white PCLTF polymer solution and the preexisting dark PCLTF were analyzed from 4000 to 650 wavelengths. The ^{1}H-NMR of the PCL-triol and the PCLTF polymer solution was recorded using a 400 MHz spectrometer, where tetramethylsilane was used as an internal reference and deuterated chloroform ($CDCL_3$) as the solvent.

In Vitro Cytotoxicity Analysis

Cell Culture. Primary human gingival fibroblasts cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; LGC Scientific, Sigma, St. Louis, MO, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Euroscience, Paa Austria), and 1% (v/v) antibiotics comprising of penicillin/streptomycin, and amphotericin (Euroscience, Paa Austria), maintained in a humidified atmosphere at 37 °C and in 5% CO_2. After the cells had reached 80–90% confluence, this confluent layer of cells became designated as the "first passage cells". Oral fibroblasts with passage not more than six were utilized for experiments. Before being used in the experiment, both the non-porous (PCLTF-GMPs scaffold before GMPs leaching) and porous (PCLTF-GMPs after GMPs leaching) scaffolds were sterilized in 70% ethanol overnight, followed by washing three times with phosphate buffered saline (PBS).

Direct Contact Test. Transwell cell culture inserts were the support devices used to study the direct contact effect of fibroblasts against the components of PCLTF-GMPs, namely the PCLTF polymer solution, the GMPs and the crosslinking agents (BP-NVP and DMT) as it is designed to produce a cell culture environment that closely resembles the *in vivo* state. First, each well was seeded with fibroblasts in DMEM at a concentration of 2 × 10^4 cells/well and incubated for 1 h. Then the mixed sterilized components of PCLTF-GMPs were placed at the bottom of each of the 24-well Transwell insert that has a membrane pore diameter of 0.4 μm. For control, fibroblasts were cultured in 24-well plates with Transwell inserts but without any specimen material. After the completion of a 1 h exposure time, where the material would have transformed from its liquid state to a solid form, the inserts were removed and cell viability were estimated.

Tests were then carried out on the porous and non-porous PCLTF-GMPs disks to determine the effect of direct cell contact with these polymerized scaffolds. The disks were preconditioned prior to this experiment by soaking in 1 mL of basic medium for 2 h. Cytotoxic evaluation was carried out by seeding human gingival oral fibroblasts onto the disk at a density of 2 × 10^4 cells/cm^2 in 300 μL/cm^2 of culture media in a 24-well plate and incubated at 37 °C, with 95% relative humidity, and in 5% CO_2.[2] A total volume of 500 μL culture media was used in each well and the media was changed on day-3 and day-7 of the culture period. A control was prepared by plating the cells with the same density, but without exposure to the disk.

The morphology of cell in contact with the non-porous PCLTF-GMPs was evaluated using FESEM (low vacuum operating mode, Quanta FEG 250, Holland). After a 3- and 7-days culture, the disks were rinsed with PBS and fixed overnight in cacodylate buffer containing 4% glutaldehyde. Cells were dehydrated in a graded ethanol series and finally dried in osmium tetroxide treatment. They were then sputter coated with gold. The cells-scaffolds were examined under a scanning electron microscope at an accelerating voltage of 10 kV.

Indirect Contact Test. The non-porous PCLTF-GMPs' extract and its leachable products were tested in accordance to ISO 10993-5 standards.[19] Three immersion times (1, 2, and 3 days) were selected to extract the non-porous PCLTF-GMPs scaffold. Briefly, the sterilized PCLTF-GMPs disks were immersed in culture media at the ratio of 1 mL of medium to 3 cm^2 of surface area and incubated at 37 °C with 95% relative humidity and in 5% CO_2. The media containing the extracts were diluted 10× and 100× with fresh culture media. Human gingival oral fibroblast culture was prepared by seeding the cells at a concentration of 2 × 10^4 cells/well in a 24-well plate. The cells were cultured to 80–90% confluency prior to the test. Once they reached the required confluency, the culture medium was removed and the fibroblasts were exposed to 100 μL of each concentration (1, 2, and 3 days) of extracts with three different dilutions (undiluted, 10×, and 100×) for 24 h.

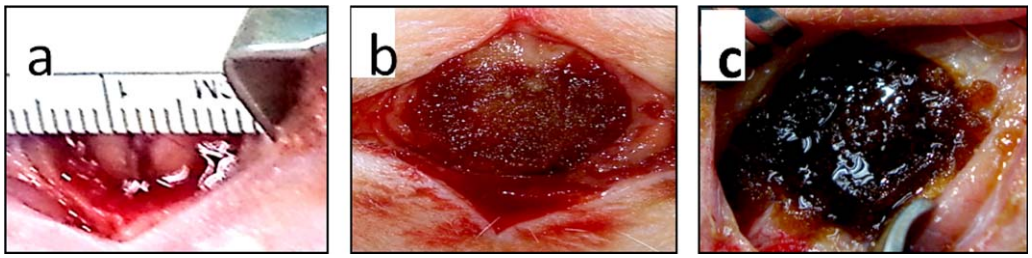

Figure 1. Interpretative view showing the (a) unfilled defect (Control), (b) defect filled with new PCLTF-GMPs, and (d) defect filled with dark PCLTF. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

For testing the cytotoxicity of the non-porous crosslinked PCLTF-GMPs degradation products, bulk scaffold degradation was performed and the cultured cells were exposed to the degradation products. The degradation process was carried out in a strong base at 37 °C, which accelerated the hydrolysis of ester.[20,21] Briefly, a PCLTF sample (0.1 g) was placed in 10 mL 1 *N* NaOH solution and secured in a water bath at 37 °C for 35 h. The sample was considered to have degraded completely when no solid material was visible in the solution. Then, the pH of the solution was adjusted to 7.4 using 1 *M* HCl and the solution was filtered through a cellulose acetate membrane filter, 0.2 μm in pore diameter. The degradation solution was then diluted with culture medium into 2×, 10×, 50×, and 100× dilutions. Sterile PBS was diluted by the same factors and used as a negative control.[21] Similar to the test for the extracts, confluent human gingival oral fibroblasts at a concentration of 2×10^4 cells/well in a 24-well plate were exposed to an aliquot of 500 μL of four different concentrations of the degradation product-containing media and then incubated for 24 h.

Cell Viability and Proliferation. Alamar blue (AB) fluorescent assay (Sigma) was used to measure cell viability and proliferation. To measure the viability of treated cells, the medium was gently aspirated from the vacated culture wells and replaced with 10% AB reagent. Cells were incubated with the AB reagent for 5 h and then the media samples were collected into a 96-well plate for absorbance detection (excitation 570 nm and emission 600 nm) using a microplate reader (Biotek ELx808 Absorbance Microplate Reader, Winooski, VT, USA). The reduction of AB assay is theoretically proportional to the growth rate and viability of cells. Thus, the mean for cell viability was determined by measuring the reduction of AB assay, and the percentage of the cell viability was determined using the following formula:

$$\text{cell viability } \% = (x/xc) \times 100 \quad (1)$$

Where x is the absorbance of the treated cell and xc is the absorbance of the control group (untreated cells). The tests were repeated at least three times to ensure reproducibility. Based on this reference, cytotoxicity responses were qualitatively rated as severe, moderate, slight, and non-cytotoxic when the percentage of cell viability were $< 30\%$, 30%–59%, 60%–90%, and $>90\%$, respectively.[22,23]

Propidium Iodide (PI) and Acridine Orange (AO) Double-Staining (Live/Dead Assay). The undiluted PCLTF-GMPs extract and the degradation products (2× dilution) were assessed qualitatively and quantitatively using acridine orange (AO) and propidium iodide (PI) double-staining according to standard procedures (Lieca attached with Q-Floro Software). Briefly, the experiment was carried out in a 25 mL culture flask. Human oral fibroblasts were plated at a density of 1×10^5 cells/mL in this flask. The cells were incubated with the undiluted extract at three different concentrations (1, 2, and 3 days) and with the 2× dilution degradation products for 24 h. Then the cells were split and transferred into centrifuge tube and centrifuged at 270 × g for 10 min. In addition, the cellular pellet was washed twice with PBS by centrifuging at 270 × g for 10 min. About 10 μL of fluorescent dyes containing AO (10 mg/mL) and PI (10 mg/mL) were added into the cellular pellet at equal volumes. The freshly stained cellular suspension was dropped into a glass slide and covered by a cover slip. The slides were observed under UV-fluorescence microscope within 30 min before the fluorescence faded off. The AO and PI were intercalating nucleic acid-specific fluorochromes (Lieca attached with Q-Floro Software, Solms, Germany), which emitted green and red fluorescence respectively. Of the two, only AO can cross the plasma membrane of viable and early apoptotic cells.[24,25]

For quantitative analysis, cell counting was completed on at least three images selected randomly for each group. The images were divided into multiple 5 × 5 grids to facilitate counting. The numbers of live and dead cells were then counted based on each grid square for the whole image. The live and dead cell counts were summed and the viability percentage was calculated as the ratio of live cells to total cells.[26]

In Vivo Study

Surgical Procedure. After characterization and *in vitro* studies, animal experiment was performed to study the biocompatibility and osteoconductivity of the PCLTF-GMPs scaffold. Six months old New Zealand White male rabbits with the average weight of 3.5–4 kg were used in this study. Each rabbit was implanted at random with either this newly modified white PCLTF-GMPs ($n = 10$), the older version of dark PCLTF ($n = 4$), or left unfilled (control) ($n = 10$) (Figure 1). For the surgical procedures, the rabbits were anaesthetized with 30 mg/kg of ketamine 100 mg/mL and 3 mg/kg of xylazine 20 mg/mL (Troy laboratories PTY. Limited, Smithfield, Australia). After hair shaving and disinfection of the operation site, full thickness a cranial critical size defect of 15 mm in diameter was drilled using trephines under cool-saline irrigation. After the placement of test materials or control (Figure 1), the wound was closed layer-by-layer with proper wound dressing applied. After surgery,

Table I. Dose and Time for the Injection of Fluorescent Bone Traces

Time	Substance	Dose	Injection (mg/Kg)
21 days	Alizarin	30 mg	3 g/100 mL + 2 g Na_2HPO_4
32 days	Alizarin	30 mg	3 g/100 mL + 2 g Na_2HPO_4
42 days	Calcein	10 mg	1 g/100 mL + 2 g Na_2HPO_4
53 days	Calcein	10 mg	1 g/100 mL + 2 g Na_2HPO_4
63 days	Tetracycline 60	60 mg	6 g/100 mL + 2 g Na_2HPO_4
73 days	Tetracycline 60	60 mg	6 g/100 mL + 2 g Na_2HPO_4
84 days	Exitus	-	-

subcutaneous administrations of meloxicam (analgesic) of a dosage of 0.3–1.5 mg/kg, and intramuscular injections of Kombitrim 1 mL/10 kg (sulfamethoxazole and trimethoprim; Kela Labratoria n.v, Hoogstraten, Belgium) (antibiotic) were given for three days. The rabbits were housed in the Animal Experiment Unit under standard laboratory conditions and allowed free access to food supply throughout the experiment period.

The following regimen of fluorochromes was administered to the animals following implantation and the labeling regime was done according to the recommendations of preceding studies[27–29] (Table I).

Investigation. After 84 days, the animals were anesthetized and sacrificed with an overdose of barbiturates (Dolethal, Pentobarbitone sodium 200 mg/mL solution, 0.7 mL/kg IV). The area of the original surgical defect and its surrounding tissues (2 mm) were removed *en bloc* for histologic and histomorphometric investigations. Histological sections were prepared using the cutting-grinding system (Exakt System, Exakt Apparatebau, Nordestedt, Germany). From each specimen, at least four sets of three serial sections were cut at intervals of 10 µm. The sections were grounded and polished to a final thickness of 100–120µm. The bone remodeling, the interface between the bone and graft, and the normal bone healing were examined using an Olympus BX-UCB fluorescent microscope (Olympus, Melville, NY) and a light microscope (Zeiss Axioplan 2, Zeiss, Jena, Germany). The areas and perimeter associated with each tracer (Alizarin, calcein, and tetracycline) individually were measured by Image Pro Multidimension Aquistion (MDA, Version 6.1.0.346) image analyzer.

Light Microscopy of Sections Stained with Toluidine Blue. To observe the remodeling process around the PCLTF-GMPs scaffold and the cells present in this region, the slides were stained with a solution of 0.5 g of toluidine blue dissolved in 100 mL distilled water and analyzed under light microscopy.

Fluorescence Microscopy. For this study, 40 unstained slides of PCLTF-GMPs filled defects and another 40 unstained slides of unfilled defects (control) were used. The areas and perimeter associated with each tracer (Alizarin, calcein, and tetracycline) were individually measured in each section by an image analyzer [Image Pro Multidimension Aquistion (MDA), Version 6.1.0.346] using a fluorescence microscope (Olympus–BxUCB, Olympus Corp., Tokyo, Japan). A filter set for red (EX: D560/40x, EM: D630/60m), green (EX: D480/30x and EM: D535/40m), and yellow (EX: ET405/40x and EM: ET550/60m) Olympus BX61 model fluorescence was used.

Data Analysis

The data analysis was done using Statistical Package for Social Science (SPSS) statistical software (Version 12.0; SPSS Inc., Chicago, Illinois, USA). Descriptive statistics were applied where appropriate. Student *t*-test was performed for comparing the results of direct contact test, while 2-way ANOVA and Mann-Whitney tests were performed for the extract and degradation products, respectively. Student *t*-test test was also performed to compare between the groups (PCLTF-GMPs and control) whereas, repeated measure Analysis of variance (ANOVA) was used for multiple comparison between the mean values of the three labels within the same group *in vivo*. The significance value was set at 95% ($P < 0.05$).

RESULTS AND DISCUSSION

Characteristics of PCLTF-GMPs

The PCL-triol contributed the main polymeric backbone, where functional groups of FCl (i.e., C=C bonds) were incorporated to synthesize a PCLTF polymer solution, rendered crosslinkable *in situ* by a free radical initiation system. The percentage yield of the PCLTF compound was 78.53%. Based on the spectra of the synthesized PCLTF polymer solution shown in Figure 2(a), the C=C stretch (at wavenumber 1641.21 cm^{-1}) and =C–H stretch (at wavenumber 3079 cm^{-1}) were not detected in the spectra of PCL-triol. However, O–H stretches with rounded broad tip were found in the spectra of both PCL-triol and PCLTF polymer solution, at wavenumber 3366.16 cm^{-1}. The presence of O–H bond indicates that the synthesized PCLTF polymer solution retained some hydroxyl groups (–OH) after reaction with FCl, hence giving it some degree of hydrophilicity to the PCLTF polymer solution. This finding was further supported by the presence of a chemical shift value for C=C–H group at 6.8 ppm as obtained from ^{1}H-NMR[30] [Figure 2(b)]. This proves that the C=C–H group had been successfully incorporated, and the PCLTF polymer solution has been successfully synthesized. This finding is similar to an earlier report that detailed the production of a dark PCLTF.[11]

The PCLTF polymer solution that was synthesized in this study was a viscous injectable product that had a long PCL hydrocarbon backbone with multiple branches of unsaturated polyesters. It could bind to a biological surface and via *in situ* crosslinking, cures into a solid structure. The GMPs (gelatin) added acts as a

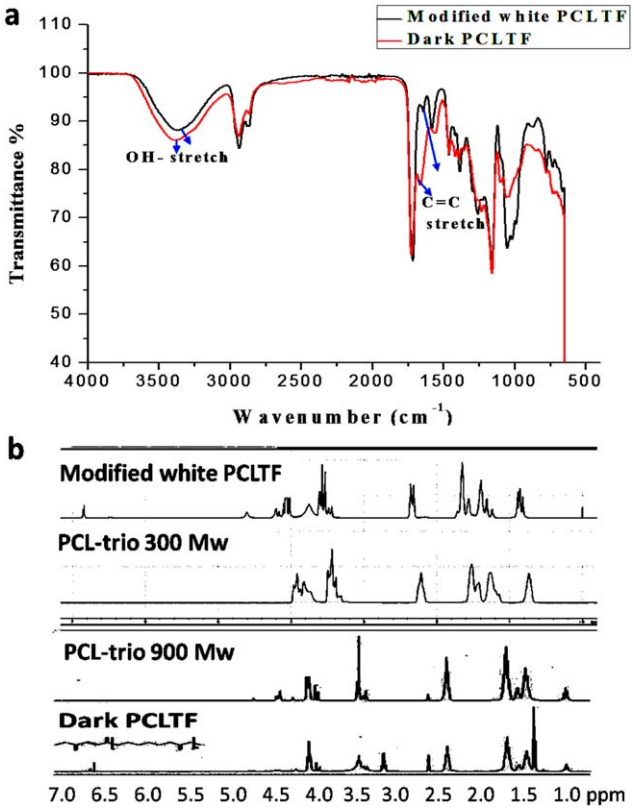

Figure 2. (a) FTIR spectra of the PCLTF polymer solution at 1641.21 cm^{-1} (C=C stretch) and 3366.16 cm^{-1} (O–H). (b) The ^{1}H-NMR spectra of the synthesized PCLTF polymer solution confirms the presence of (CH=CH) at 6.8 ppm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

porogen to help create a porous structure with increased microchannels and interconnectivity for cell adhesion. This process became possible once it was leached out *in vivo* through enzymatic degradation i.e., through the action of collagenase. The GMPs play an important role to facilitate cell function and initial cell survival, as it has a high capacity of fluid uptake. The addition of GMPs as a cell adhesion component to this current PCLTF polymer solution, which has a long PCL hydrocarbon backbone, may provide a good surface for cell attachment. Furthermore, gelatin is an excellent extracellular matrix equivalent due to its highly hydrated nature,[31] allowing it to be delivered in a liquid form that can be left to solidify *in situ*.

It is highly important that solidification takes place within a clinically relevant time frame so that the material is localized at the point of interest. The solidification should be selective so as not to cause necrosis to the surrounding tissue due to excessive heat emission, yet is tolerable to the encapsulated cells and/or any potentially sensitive molecules. Moreover, it should have a fast solidification process that also allows homogeneous cell dispersion within the gelatin matrix.[32] In this study, the hardening process of PCLTF-GMPs was relatively fast, and was able to achieve significant crosslinking *in situ* within an acceptable time frame ~5–7 min at room temperature and with 26 °C heat emission that is lower than the body temperature. This is an improvement when compared to the 15 min recorded for the dark PCLTF.[11]

Cytotoxicity Test

The other goal of this study was to evaluate the cytocompatibility of this novel injectable scaffold (PCLTF-GMPs) in its different life span i.e., in injectable form (during polymerization), as PCLTF-GMPs before and after GMPs leaching, and of its extracts and degradation products. In clinical situation, once a scaffold is placed into an extraction socket, the biomaterial will first come into contact with the soft tissue (gingiva) before bone regeneration can take place within the scaffold. To ensure that the product is biocompatible with the soft tissue, it needs to be tested against human gingival fibroblasts similar to the process done in an earlier study.[11]

The measurement of cytotoxic effects can be determined based on the presence/absence and the amount of metabolically active cells. This method also assesses cell growth. In general, cells will not grow in a cytotoxic environment. Alamar blue assay was studied for this purpose, with the assumption that continued cell growth will result in a reduced environment (turns pink color) while the inhibition of growth will maintain an oxidized environment (maintains blue color). Hence, in this study, cell proliferation was evaluated by comparing the color change in response to the chemical reduction of growth medium immersed with a test sample versus non-exposure in the control group. The reduction of the Alamar blue is theoretically proportional to the growth rate and viability of cells. Based on ISO 2009, a biomaterial that causes the inhibition of cell growth of more than 60% as compared to the control is defined as toxic material.[19,22,23]

Direct Contact Test

Fibroblasts showed slight reduction of growth compared to the control when tested in direct contact test against the *in situ* crosslinking-released components, after 1 h of polymerization/hardening exposure. Since polymerization needs between 7 min and 1 h to form a completely solidified scaffold, the duration of incubation was set at 1 h. The inhibition of cell growth in the crosslinking-released components of the PCLTF-GMPs was found to be 11.5 ± 1.6%. Hence, this biomaterial can be considered to have slight cytotoxicity during hardening. However, as the main components of PCLTF-GMPs (PCL, FCL, and GMPs) are acknowledged as biocompatible materials, the authors are of the opinion that this slight degree of cytotoxicity relates mainly to the reagents used. These reagents were expected to eventually be either consumed completely during the crosslinking reaction (for BP) to form a non-toxic polymer (polyNVP) after hardening, or be present in very small amount (for *N,N*-dimethyl-*o*-toluidine) where their toxic effect became negligible.[33–35]

In scaffold contact test (after hardening), changes in cell morphology were detected with the use of inverted light microscopy. The cells grown on the PCLTF-GMPs porous scaffold showed normal cellular morphology features characterized by spindle-shaped fibroblast morphology. Corresponding to this, fibroblast proliferation increases exponentially with the increase in time.

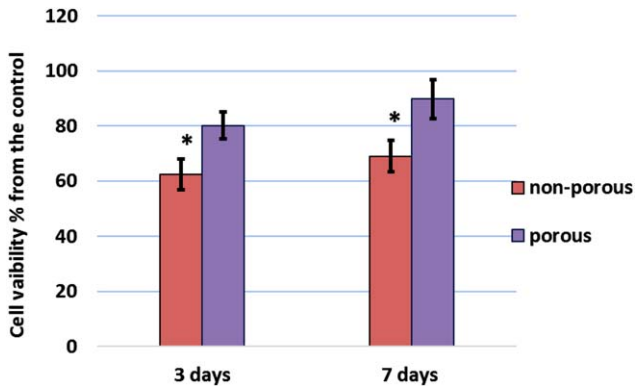

Figure 3. Porous scaffold showed a significantly higher percentage of viable cell when compared to the non-porous scaffold (*$P < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Alamar blue assay showed that fibroblasts remained viable on the porous and non-porous PCLTF-GMPs scaffold throughout the culture period and there was a significant increase in the Alamar blue reduction over the 7 days of culture [$P < 0.05$; $P = 0.014$ (porous), $P = 0.034$ (non-porous)]. In addition, the mean difference in AB reduction between the test and control was further reduced at 7 days compared to 3 days. This showed that the test materials were biocompatible. Figure 3 shows a significantly higher percentage of cell viability in the porous scaffold than the non-porous product. This observation suggests that porosity increases cell contact surface area, hence permitting more cells to fill the porous void space and structure.

Figure 4 shows the FESEM cellular morphological appearance of fibroblasts cultivated on the porous PCLTF-GMPs scaffolds for 3 and 7 days. Even distribution of cells was observed on the scaffold surface, together with good cytoplasmic attachments. This finding supports the result of the direct contact test, where the scaffold was found to have a favorable surface for cell attachment.[36]

Indirect Contact Test (Extract and Degradation Products)

The scaffold was immersed for three different immersion times, i.e., 1, 2, and 3 days to obtain three different concentrations of PCLTF-GMPs extract (leachable) products that were then subjected to indirect contact test. Again, Alamar blue assay was used to evaluate cell viability of these three different concentrations of non-porous PCLTF-GMPs extract subjected to three different dilutions, namely the undiluted form, 10× dilution, and 100× dilution. In their undiluted form, no significant difference was noted for the three different concentrations of extracts, whereby all of them showed moderate cytotoxicity. At 10× dilution, there was also no significant difference between the first concentration and the second concentration, both of which showed slight cytotoxicity. There was however, a significant difference noticeable between the second and third concentration at this dilution. At 100× dilution, there was significant differences between all three different concentrations ($P < 0.05$), but with second and third concentration showing non-cytotoxic effect. The authors are of the opinion that the significant increase in Alamar blue reduction at 10× and 100× dilutions resulted from the presence of a higher concentration of GMPs that became dissolved and released from the scaffold into the extract, thereby enhancing cell growth and proliferation. In all, there was evidence that cells continued to grow at all three concentrations of extracts. For the first concentration, the reduction values significantly increased from the undiluted form to 10× and 100× dilutions, and when compared to the control group ($P = 0.01$). It showed a reduction value comparable with the control at 100× ($92.45 \pm 5.4\%$) of the second concentration, and at 10× ($94.9 \pm 6.2\%$) and 100× ($102.6 \pm 5.1\%$) dilutions of the third concentration; the later showed evidence of enhanced cell growth (Figure 5). More diluted extracts of all three different concentrations showed significantly ($P < 0.05$) higher reduction values when compared to the undiluted and 10× diluted forms; this reflected higher cell viability. This observation suggests that the toxicity effect became reduced with the dilution of extracts, as shown by the change of moderate cytotoxic effect in the undiluted first concentration to become comparable and growth enhancing in the third concentration of 100× dilution. However, the lowest cell viability found in the undiluted first concentration ($50.0 \pm 3.9\%$) is suggestive of moderate cytotoxicity, which is considered an acceptable cytotoxic value according to the ISO10993-5 standard. The

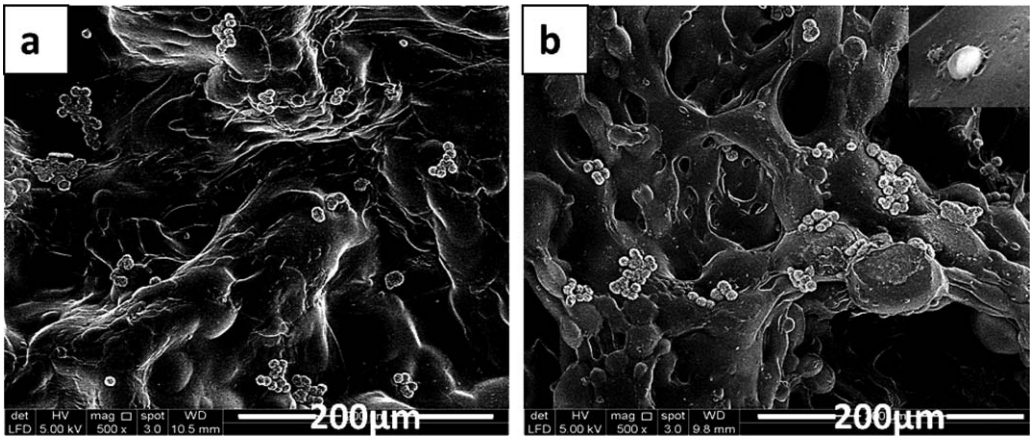

Figure 4. Representative FESEM micrographs of seeded porous PCLTF-GMPs scaffolds cultivated for (a) 3 days and (b) 7 days with fibroblast cells (scale bar, 200 µm). The insert on the upper right corner shows a cell attached on the PCLTF-GMPs surface, seen at a higher magnification (scale bar, 50 µm).

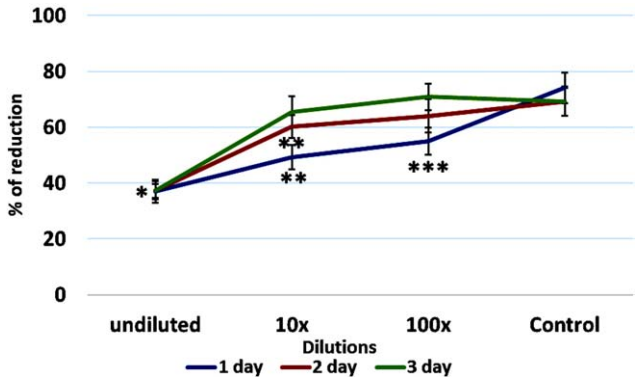

Figure 5. The results for the indirect contact test of the PCLTF-GMPs extracts. The percentage of Alamar blue reduction assay shows cell viability of oral fibroblasts after exposure in the extracts for 24 h. $^{*}P < 0.05$ between the dilutions of the three concentrations and the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

authors are of the opinion that this moderate cytotoxic effect resulted mainly from various possible substances, such as the accelerators, that leached out of the polymer. These substances often have low molecular weight and exhibit varying degree of physiological activity and cell toxicity. Although the cytotoxic effect of the undiluted extracts would be moderate initially, in clinical condition, this effect might reduce with time whereby the concentration of extracts would become diluted by the surrounding tissue fluid to become nontoxic. This postulation has been confirmed by the results obtained with different dilutions that are supposed to mimic the natural physiological buffering effect of tissue fluids. In addition, during the cross-linking process of the polymer, the constituents continue to integrate into the network, resulting in minimal diffusion of the leachable products out of the network.[37–39] In summary, dilution and the decrease of free leachable extract products coupled with the increase of the GMPs in the solution contribute to an increase in the percentage of viable cell.

The cells cultured showed a normal spindle-shape cellular morphology that is characteristic of fibroblast. An increase in cell proliferation was observed in the more diluted form of extract. This finding supports the result observed for the extract's indirect contact test using Alamar blue reduction.

In general, products formed during the degradation of bioresorbable polyester materials are considered non-toxic and are excreted naturally from the human body even though hydroxyl acids may potentially induce a decrease in pH,[40,41] leading to cell apoptosis.[42] The body will normally be able to buffer this acidity and prevents the formation of local acidic environments in the body.[31] At this juncture, the authors are unable to confirm this assumption as the test was only done *in vitro*.

The non-porous form of scaffold was designated a control because it was made up of the whole components of scaffold i.e., PCLTF-GMPs before the porogen was leached out. The Alamar blue reduction for the indirect cytotoxic test of the non-porous PCLTF-GMPs degradation products is shown in Figure 6. Although it revealed slight toxicity (88.94 ± 7.4%) at 2× dilution, it was deemed comparable, non-cytotoxic, and was able to enhance cell growth at 10×, 50× and 100× dilutions ($P > 0.05$) based on the ISO guide. This finding is in accordance with that observed for the extract test where the cytotoxicity decreased when the extract product became diluted. This finding is similar to that reported for the dark PCLTF.[11] Although both results showed moderate cytotoxicity for the undiluted extract and slight toxicity in the more concentrated degradation products (i.e., 2× dilution), one should note that the *in vitro* culture condition does not represent a homeostatic condition like that *in vivo*. As highlighted in the previous report,[11] the *in vitro* experiment lacked of defined lymphatic mechanisms, which have a strong impact on the precision of toxic elimination.[22,43]

Live/Dead Assay

Equally important in the assessment of the biological reaction of a biomaterial is determining the presence of cell damage or death. There are two different pathways that lead to cell death, namely apoptosis and necrosis, both of which may be activated by non-biocompatible biomaterials. The way and extent of the apoptosis and/or necrosis could reflect their degree of biocompatibility.[44]

In the live/dead assay, apoptosis (blabbing and nuclear margination) was noticed in the first concentration of treated cells. There was a presence of red color due to the binding of acridine orange (AO) to denatured DNA [Figure 7(a)]. However, also seen was the presence of viable cells with intact cell wall shown as green color under fluorescence, which was comparable to the control. The proliferation and cell viability in the second (63.3 ± 2%) and third concentrations (68.2 ± 2.2%) were higher than the first concentration (50.2 ± 6.3%). These findings confirm the result observed in the Alamar blue assay where high concentrations of GMPs were noted to enhance cell growth.

Altogether, most cells were viable (85.2 ± 3.6%) with regards to the 2× degradation products of the PCLTF-GMPs as shown in fluorescent green color with intact cell wall. Furthermore, the

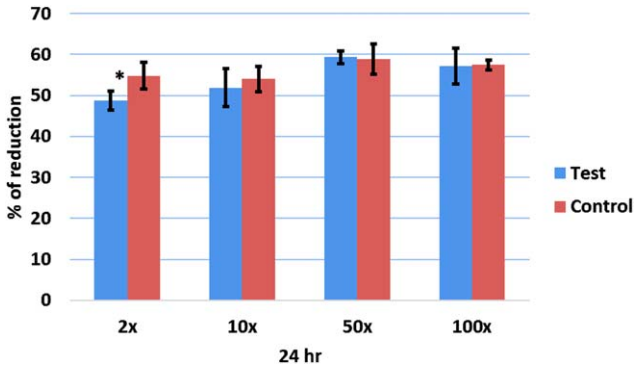

Figure 6. The indirect contact test of the degradation product of PCLTF-GMPs. It shows the percentage of cell viability after the exposure of oral fibroblasts to the degradation product over a period 24 h. The reduction percentage of the 2× dilution group was significantly less than the control ($^{*}P < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

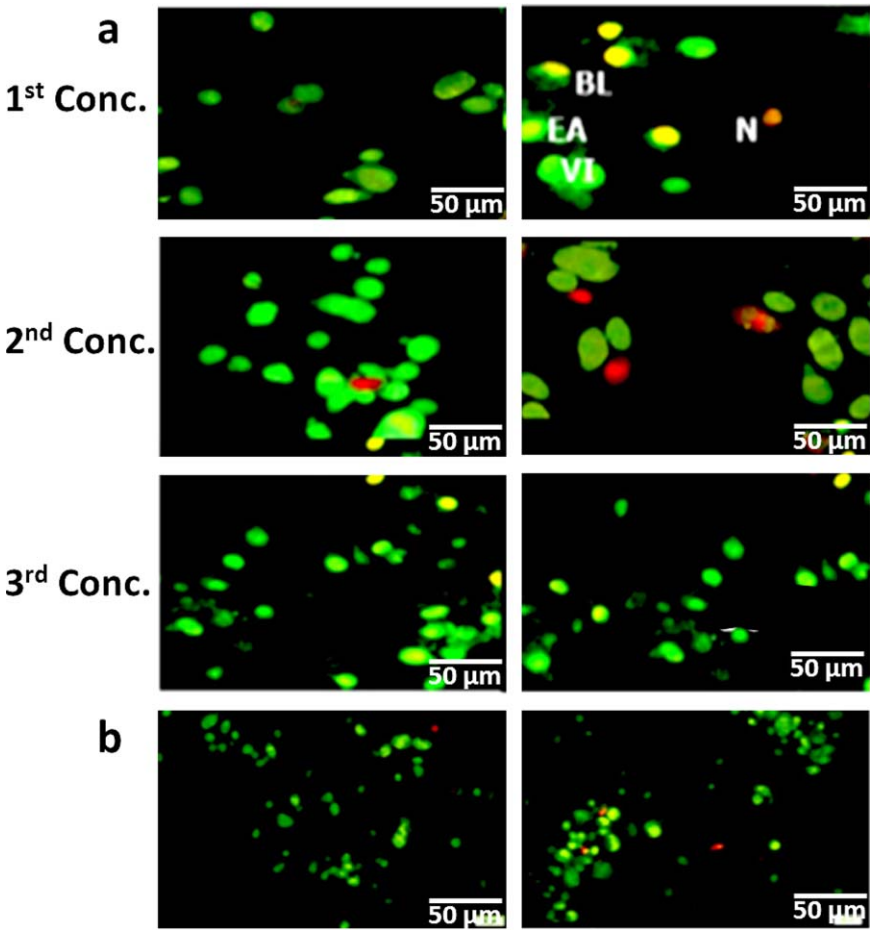

Figure 7. The fluorescent micrographs of AO (green) and PI (red) double-stained fibroblast cells. VI represents viable cell, EA represents early apoptosis, and BL represents late apoptosis. The untreated cells (left) showed normal structure without prominent apoptosis and necrosis after 24 h. Early apoptotic features were seen as intercalated AO (bright green) amongst the fragmented DNA in (a) first concentration of extract, which became less prominent in the third concentration (right). (b) 2×-degradation product group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

proliferation and growth of these cells was quite similar to that of the control [Figure 7(b)].

In Vivo Study

In earlier studies, porous scaffolds fabricated from PCLTF polymer solution that was based on the salt porogen showed good biocompatibility *in vitro*,[11,45] and the current study confirmed that the modification made did not in any way make the current product any inferior. However, previously no further *in vivo* studies were done to investigate the biocompatibility of the PCLTF-salt products using animal models. This current study, thus further investigated this effect, by using it as a positive control, against which the newer modified PCLTF-GMPs was compared with.

Macroscopical and Morphological Assessment. Clinically, the defects in all rabbits (n = 4) filled with dark PCLT-salt porogen material were associated with local inflammation within the first three days that then progressed to suture opening and dislocation of the graft with pus discharging at the end of the 10 days. These rabbits were sacrificed and no further work was undertaken on this material. The authors think that this complication is most likely the result of local toxicity caused by the high osmolarity of salt in the implanted site. However, the healing of the PCLTF-GMPs implanted defects was uneventful. No signs of inflammation or infection were encountered. This is one of the hallmarks of biocompatibility for the newly modified PCLTF-GMPs. In addition, the bone in most of this white PCLTF-GMPs filled defect was thick and hard and completely covered the defect surface. Although some defects showed the presence of the main graft material in the central part after 84 days, it appeared to be fused to the newly formed bone at the margins. In comparison, in the unfilled defect there was a collar of soft tissue with minimal hard tissue fused to the margin.

Light Microscopic Finding. As the dark PCLTF implanted sites were inflamed and the grafts were dislodged, a decision was made to sacrifice the rabbits in this group at day 10, and the entire wound defects in the experiment sites were harvested *en bloc*, fixed in 4% paraformaldehyde, decalcified, processed, and stained with hematoxylin and eosin (H&E) for histologic analysis. It revealed that there was no sign of new bone formation at the margin of the defect adjacent to the necrotic bone. Instead,

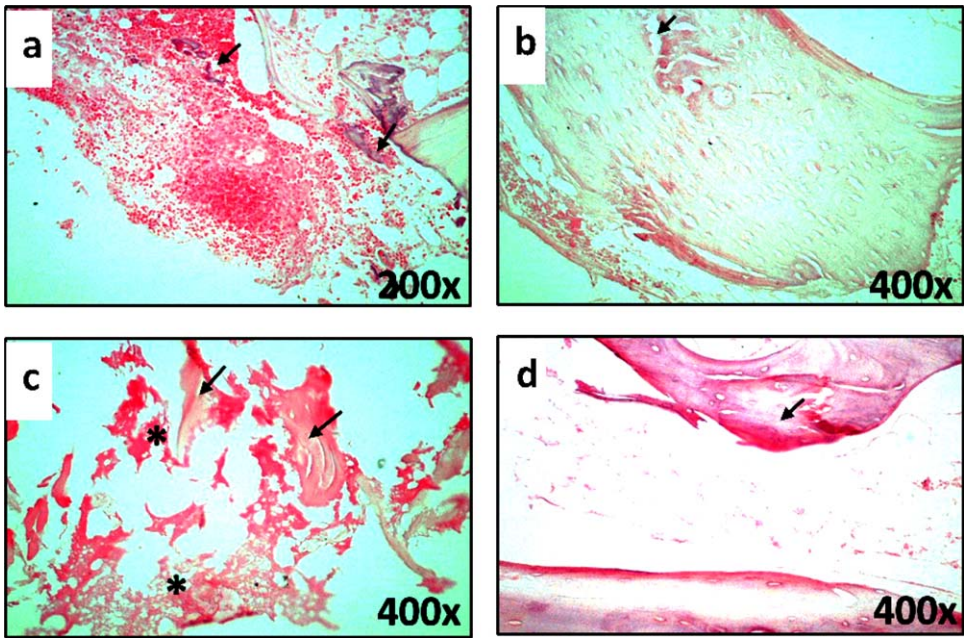

Figure 8. Photomicrograph of the dark PCLTF filled defect at day 10 of experiment; (a) Necrotic bone at the margin of the defect (arrow) and giant cell (head arrow) (HE, 200×), (b) internally resorbed lacunae that resulted from the union of several lacuna (arrow) (HE, 400×), (c) fragments of the PCLTF-salt porogen (*) and the bone (arrow) with no sign of contact or osteoid formation, and (d) dead bone and no trabecular bone (dipole) between the cortical bones (arrow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

there was extensive resorption and the presence of non-vital bone with empty lacunae, vasodilation, and some giant cells [Figure 8(a,b)]. The implanted graft material has separated from the bone [Figure 8(c)]. The trabecular bone in the cancellous areas (diploe) was resorbed [Figure 8(d)].

However, unstained sections for the control and PCLTF-GMPs implanted sites showed new bone formation, which grew and developed from the marginal defects toward the center (Figure 9). The cavity (subsequently termed 'defect') implanted with PCLTF-GMPs healed as an in growth of woven bone beginning from the defect margins and as formations of bony island within the defect area. This appearance seems to suggest that the neighboring small islands will eventually merged to form large islands and close the defect over time. PCLTF-GMPs grafts were not rejected and were incorporated within the newly deposited bone.

Bioactive materials are known to bond to living bone via the formation of an apatite layer on their surfaces.[46] In the present study, micrographic toluidine blue stained study showed the integration of the PCLTF-GMPs with new bone deposition on its entire surface. This confirmed that bone growth had occurred preferentially near the PCLTF-GMPs scaffold as well as on its surface, with in-growth into its pores. Based on these finding, two types of interfaces were recognized and characterized. The first was visible bone contact with the white PCLTF-GMPs graft and the second was interposition of new bone on the surface of the graft. Furthermore, graft-bone bridging processes were present in PCLTF-GMPs graft filled defects as seen by bony extensions from the peripheral in-growth towards the center, attempting to close the defect. This appearance was noted in most of the PCLTF-GMPs filled defects. The presence of some remaining graft at the center of the defect at day 84, embraced by bone and interconnected by bone trabecular and bony island near the surface of PCLTF-GMPs is suggestive of the osseointegration property of this material. The compatibility and osteoconductivity of the PCLTF-GMPs scaffold could be seen by the presence of progressive bone neoformation induced by osteoblasts and bone remodeling throughout the experimental site. Osteocytes and osteon could be routinely identified in the control and grafted sites, and some of them were close to the graft surface. There are remnants of the darkly-stained, primary trabecular scaffold covered by new bone lamellae, as shown in Figure 10.

Overall, histological evaluation showed that PCLT-GMPs grafts were well accepted and incorporated within the newly deposited bone. New bone formed at the margins of the defects and at the same time, diffused towards its center. Most of the PCLTF-GMPs scaffold were gradually resorbed by multinucleated osteoclast-like cells and replaced with new bone. These features are a confirmation of osteoconductive property of PCLTF-GMPs. Moreover, cortical organization, as seen by the presence trabecular projections with maturation of lamellar bone, and growing thickness at the margin of the defect was comparable with the original bone structure of the region. These observations are no difference to that those observed for other biocompatible materials.[47] Lastly, the remodeling process around in PCLTF-GMPs filled defect was higher than that in the unfilled defect. This indicates the conductive behavior of PCLTF-GMPs in aiding in bone healing when compared with the dark PCLTF and/or unfilled defects.

Fluorescence Microscope Findings. Fluorescence microscopic analysis of critical size bone defect area implanted with PCLTF-

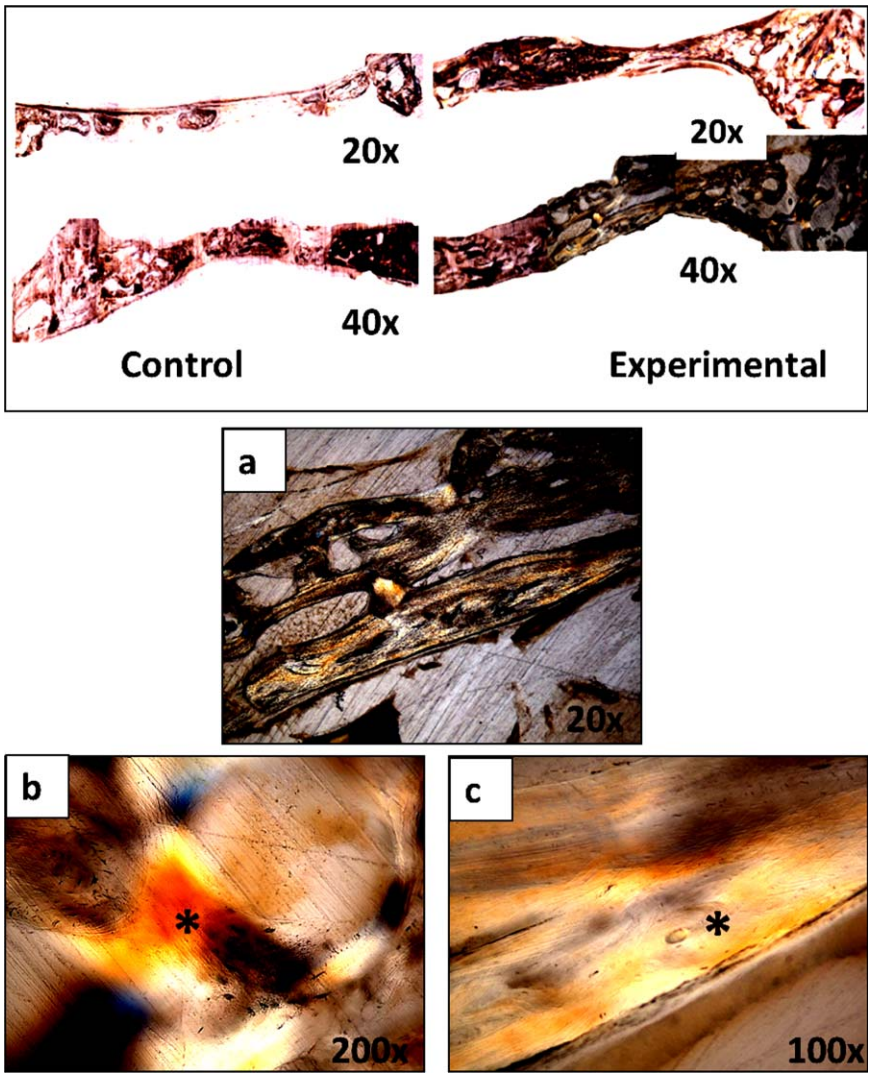

Figure 9. Photomicrograph of the control and PCLTF-GMPs filled defect (experimental) at 84 days, (a) The PCLTF-GMPs scaffold (*) filled defect showed direct bone/graft contact (b,c) osteocytes and osteon near the scaffold surface (arrows). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

GMPs at day 84 also showed an intense bone deposition and remodeling, and osteoconduction of the PCLTF-GMPs scaffold. It was possible to observe that areas marked by tracer activity were more intense in the experimental site than the control site (Figure 11). Similar to other reports, mineralized bone filling the defect appeared as areas stained with fluorochromes.[48] The colors of these fluorochromes represented the regions of calcium precipitation at different moments of tissue mineralization.[49]

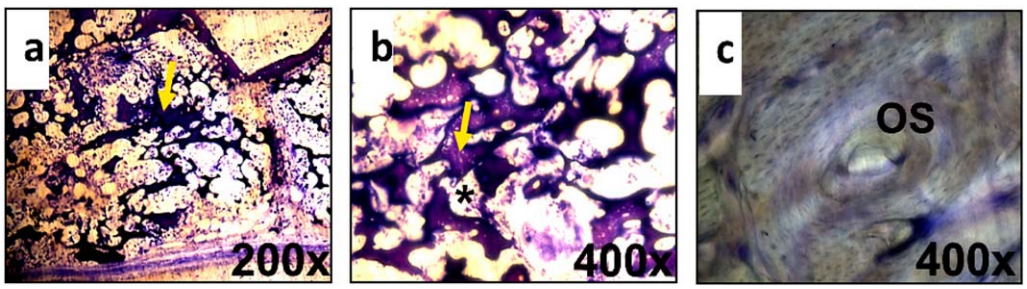

Figure 10. (a,b) Photomicrograph of PCLTF-GMPs filled defect (experimental) at day 84, showing direct bone/scaffold contact where the scaffold was completely embraced by bone and interconnected by bone trabecular (arrows); osteoblast and osteoclast are present near the scaffold surface (*). (c) There is osteon formation (OS). Osteoblasts deposited osteoid around the scaffold and an extensive bone resorption around remnants of partly degraded graft (Toluidine blue stain). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

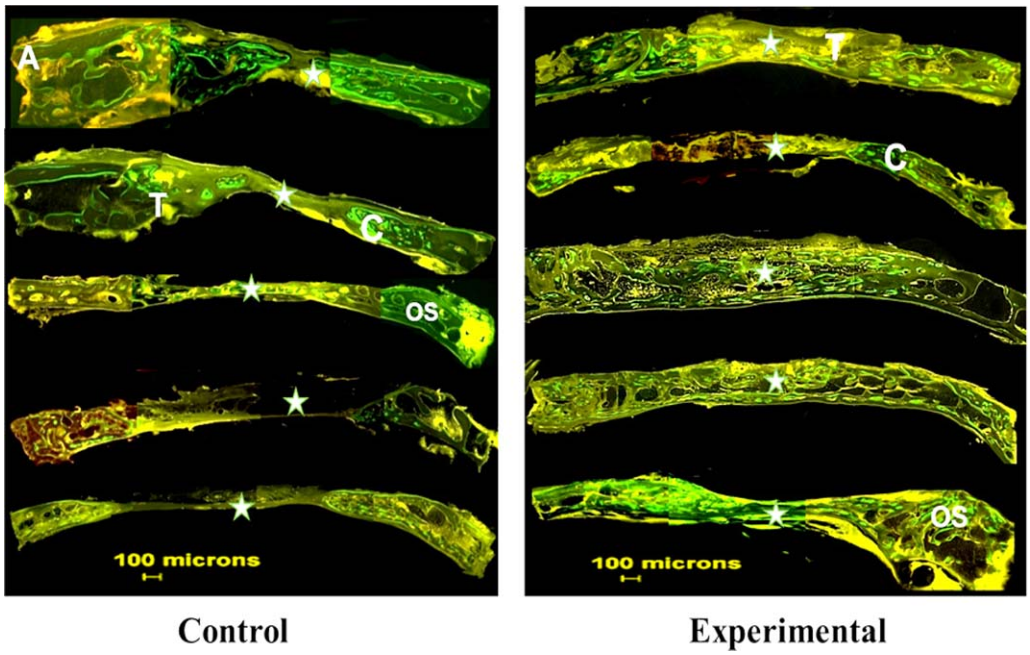

Figure 11. Fluorescence photomicrograph of the PCLTF-GMPs filed defects (experimental) and unfilled defect (negative control) (as seen using a combination of three fluorochromes). Different colors revealed newly formed bone that was stained by three markers. Alizarin (A) red color, calcein (C) green color, tetracycline (T) yellow color, and osteon (OS). This photomicrograph shows different bone thickness and bone bridging in the central part (*) of the defects of both groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Studies have shown that extensive calcification of new bone occurred in large part after 3 weeks post-implantation.[50,51] This is because of the fact that the most intense cellular reaction occurs during the first 3 weeks in a bony defect, but with little calcium deposition during this period. In another word, the defect is first bridged by a trabecular framework consisting of primitive woven bone. Hence, in this study, the fluorochrome labeling, used for evaluation of bone growth, started at 3 week post-surgery. It is expected that in the following weeks 5–8, there would be a reduction in the numbers of cells in these areas, but an increase of calcium deposition.[50,52] In this current study, the trabecular woven bone was marked first by Alizarin red (3–4 week) and was later replaced by calcein green labeling to show the highest mean value (6–8 weeks) resulting from an increasing calcium deposition and incorporation of the graft with newly deposited bone. Studies reported that bone remodeling is very active after 8 weeks of healing and is presented with diverse degrees of bone maturation and also irregular new bone formation in the marrow cavity. In this study, tetracycline labeling was done for this period, with the mean percentage value expected to decrease when compared to the percentage observed during the calcein period.

Fluorochromes are usually administered on two occasions separated by an interval of several days to enable researchers distinguish the layers of bone formed. The presence of well-resolved double labels indicates that normal bone mineralization was actively occurring over the labeling interval.[53] As observed, fluorescence from both calcein and tetracycline appeared as distinct double line separated by a layer unlabeled bone while alizarin was seen over a scattered area [Figure 12(a)]. Tetracycline was found to concentrate more in the lamellar bone as distinct yellow–green double lines at the margins of the defect and also directly towards the central area where it can be found as scattered areas of labeling. The presence of osteons in different colors, noted near the graft, confirmed that osteogenesis occurred during the entire period of grafting. Osteons were found in many areas of experimental defect [Figure 12(b)]. This is suggestive of the continuity of bone deposition and bone healing in the PCLTF-GMPs filled defects.

The distribution of the new bone deposit was represented by the mean of all areas and perimeter of polyfluorochrome labeling measured in each slide. The bone tracers showed that there were different stages of bone growth around and within the graft, as shown by the deposition of the polyfluorochrome labeling of the newly formed bone apatite. Figure 13 shows a comparison in the mean percentage of area and perimeter of newly deposited bone in the control and experiment group (PCLTF-GMPs). It showed that the highest mean value of the area and perimeter of the newly deposited bone was within the duration of the calcein administration period in both control (area $= 5.4 \pm 2.2$, perimeter $= 44.0 \pm 8.3$) and the experiment groups (area $= 10.5 \pm 4.2$, perimeter $= 58.6 \pm 5.5$). This was followed by tetracycline and then alizarin staining period, corresponding to the timing bone formation as described earlier. The thickness of the newly formed bone as measured using image analyzer (Bersoft image measurements) showed that bone thickness of the experiment group was significantly greater than the control at the center but not at its margins.

Independent *t*-test was performed to verify possible differences in the amount of newly formed bone between the experimental and control site. There was a significantly higher bone trace

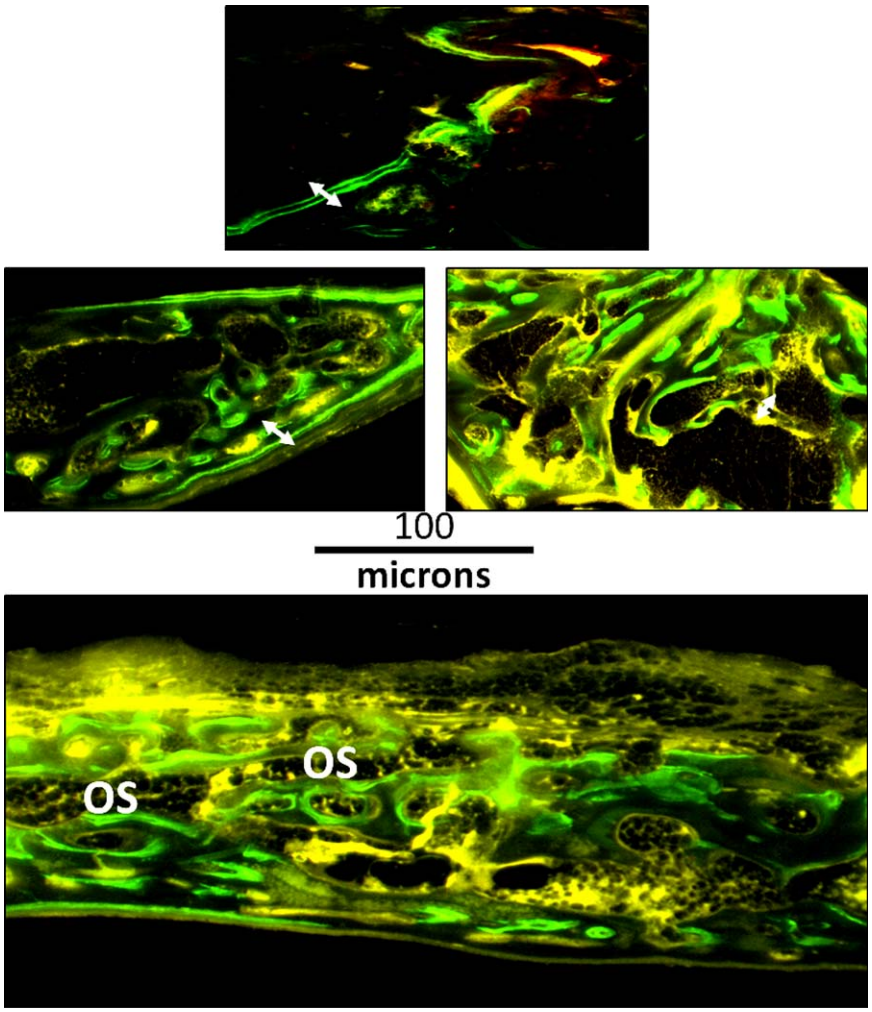

Figure 12. Fluorescence photomicrograph showed both calcein and tetracycline distinct double lines separated by a layer unlabeled bone (arrow) (a), and the presence many osteons (OS) in different colors (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

quantity during calcein staining period in the experiment groups than the control group ($P = 0.003$ for area and $P = 0.001$ for perimeter). In addition, repeated measure of ANOVA was performed to verify possible differences in the rate of bone formation during the periods of alizarin, calcein, and tetracycline administration among the two groups. Based on estimated marginal means using Bonferroni multiple comparison test, it was possible to identify a significant differences between the amounts of the new bone deposition within the groups at different periods of markers administration. In the control group, multiple comparison tests showed a significant difference between alizarin and calcein, and between alizarin and tetracycline administration, but no significant difference was noted between the periods of calcein and tetracycline administration ($P > 0.05$). In comparison, there was a significant difference with regards to the area of deposited bone for all the administration periods in the experiment groups ($P < 0.05$, $P = 0.01$). With regards to the size of perimeter of deposited bone, it was significantly greater in the PCLTF-GMPs defect than that of the control at all the administration periods ($P < 0.05$, $P = 0.01$).

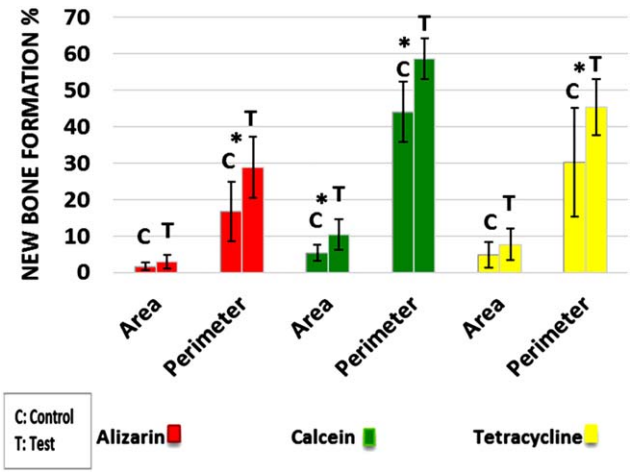

Figure 13. The mean percentage difference of the three fluorochromes in the area and perimeter of the new deposited bone in the control and experimental group (PCLTF-GMPs). C (control) and T (PCLTF-GMPs) filled defect ($^*P < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Changes in bone formation and remodeling on the PCLTF-GMPs scaffold surface are reflected in the sequence of labels administered. The presence of a few scattered areas of alizarin in the marginal area of the defect was related to primary callus formation while the presence of calcein was related to the early bone deposition that is represented by the presence of primary osteon. The presence of tetracycline labeling was related to the remodeling process and the presence of newly formed bone in the center of the defects. The low quantity of alizarin detected at the defect margins suggested that it might intermittently be replaced by subsequent calcein labels whereas, the presence yellow label over the entire graft indicated active remodeling and bone formation until the end of the observation period of the 84 days. The intense presence of calcein fluorescence marker indicates that there was adequate bone formation following the implantation of PCLTF-GMPs. This finding confirmed good maturation of tissue at different times of study. Our findings are consistent with previous studies that emphasized good healing of the bone defects.[50,51]

In all, the results obtained showed that this modified PCLTF-GMPs is biocompatible in both *in vitro* and *in vivo* tests. It does not critically affect fibroblasts proliferation and differentiation. Even at its highest concentration, the undiluted extract with 3 days immersion time and 2× dilution of degradation product showed only moderate and slight cytotoxic effect when compared to control. The cytotoxic effect was reduced, subsequently became non-toxic, and even enhanced cell growth with further dilutions. The evaluation of biocompatibility and osteoconductivity PCLTF-GMPs showed superiority against the positive control, namely the older version of dark PCLTF-salt polymer. No adverse reactions such as inflammation were observed, and it was proven to be osteoconductive and promote progressive bone remodeling during the entire period of bone healing. The presence of salt in the dark PCLTF, however, caused local toxicity in the implanted site and subsequent rejection of this material. The complete closure of the critical size bone defect filled with the PCLTF-GMPs as compared to the control further supports the favorable biological properties displayed by this biomaterial in a clinical situation.

In summary, the authors have successfully developed a novel polymeric material (white PCLTF-GMPs) that has the potential to be used as a biocompatible, *in situ* crosslinkable bone scaffold to promote bone regeneration. The main novelty of this product is its injectability, the ability to crosslink *in situ* without any manipulation and the ability to solidify within an acceptable clinical time frame. Beside the potential to being a tissue engineering scaffold, this novel product also may has the potential to become an efficient vehicle for *in situ* delivery of bone morphogenic proteins. Future investigations include studying its potential use on delivery a carrier system will be needed.

CONCLUSIONS

A modified PCLTF-GMPs has been successfully synthesized. The results of cytotoxicity test of this injectable novel scaffold *in vitro* suggested that this biomaterial was cytocompatible. It showed no evidence of critical cytotoxic effect during material-cell contact test, from the starting point of its life (before crosslinking) until the end-point of its life (degradation products). This white PCLTF-GMPs biomaterial was biocompatible when implanted *in vivo* in its injectable form as no adverse reaction was verified near the critical defect area. Moreover, calcified bone tissue was consistently observed to form on its surface and this new bone growth continued to happen during the entire period of the tracers' activity. Appositional bone formation that simultaneously replaced the PCLTF-GMPs graft was mostly accomplished at day 84th with the graft material that showing complete osseointegration with continued resorption and replacement by new bones. All these promising results have shown that the modified white PCLTF-GMPs porous scaffold is a potential bone substitute candidate for reconstructing bone defect.

ACKNOWLEDGMENTS

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