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### Authors

Gould, Toby WA  
Birchall, John P  
Mallick, Ali S  
[et al.](#)

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## Development of a porous PLGA-based scaffold for mastoid air cell regeneration

Toby W. A. Gould, BSc<sup>a</sup>, John P. Birchall, MD<sup>b</sup>, Ali S. Mallick, MRCS<sup>b</sup>, Tamara Alliston, PhD<sup>c</sup>, Lawrence R. Lustig, MD<sup>d</sup>, Kevin M. Shakesheff, PhD<sup>a</sup>, and Cheryl. V Rahman, PhD<sup>a</sup>

Cheryl. V Rahman: cheryl.rahman@nottingham.ac.uk

<sup>a</sup>Division of Drug Delivery and Tissue Engineering, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

<sup>b</sup>National Institute for Health Research Nottingham Hearing Biomedical Research Unit, Department of Otolaryngology Head and Neck, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, UK

<sup>c</sup>Department of Orthopaedic Surgery, University of California San Francisco, San Francisco, CA 941143, US

<sup>d</sup>Department of Otolaryngology, Head and Neck Surgery, University of California San Francisco, San Francisco, CA 941143, US

### Abstract

**Objective**—To develop a porous, biodegradable scaffold for mastoid air cell regeneration.

**Study Design**—*In vitro* development of a temperature-sensitive poly(DL-lactic acid-*co*-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) scaffold tailored for this application.

**Methods**—Human mastoid bone microstructure and porosity was investigated using micro-computed tomography. PLGA/PEG-alginate scaffolds were developed and scaffold porosity was assessed. Human bone marrow mesenchymal stem cells (hBM-MSCs) were cultured on the scaffolds *in vitro*. Scaffolds were loaded with ciprofloxacin and release of ciprofloxacin over time *in vitro* was assessed.

**Results**—Porosity of human mastoid bone was measured at 83% with an average pore size of 1.3mm. PLGA/PEG-alginate scaffold porosity ranged from 43–78% depending on the alginate bead content. hBM-MSCs proliferate on the scaffolds *in vitro*, and release of ciprofloxacin from the scaffolds was demonstrated over 7–10 weeks.

**Conclusion**—The PLGA/PEG-alginate scaffolds developed in this study demonstrate similar structural features to human mastoid bone, support cell growth and display sustained antibiotic release. These scaffolds may be of potential clinical use in mastoid air cell regeneration. Further *in*

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Correspondence to: Cheryl. V Rahman, cheryl.rahman@nottingham.ac.uk.

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**Conflict of interest:** None

**Level of evidence:** N/A

*in vivo* studies to assess the suitability of PLGA/PEG-alginate scaffolds for this application are required.

## Keywords

Scaffold; PLGA; alginate; mastoid; ciprofloxacin

## 1. Introduction

The mastoid portion of the temporal bone is composed of interconnected air-filled chambers. This mastoid air cell system is reported to have a number of important functions including sound conduction, maintaining pressure regulation, gas exchange, material secretion, waste excretion and middle ear cavity aeration<sup>1-3</sup>. Following mastoidectomy, mastoid obliteration procedures are focussed on filling the mastoid cavity and do not repair the mastoid bone structure. However, the only way to restore the vital functions of the mastoid is to fully regenerate the air cells within the bone.

Various polymers have been investigated for use as scaffolds in bone regeneration, including poly(DL-lactic acid-co-glycolic acid) (PLGA), which has a long history of use as a degradable implant material<sup>4</sup>. As previously described by Dhillon et al, blending PLGA with a plasticiser such as poly(ethylene glycol) (PEG) allows the production of temperature-sensitive particles with a reduced glass transition temperature ( $T_g$ ) of 37°C<sup>5</sup>. When these particles are mixed with a carrier solution at room temperature a paste is formed that can be moulded into any size or shape. The paste then hardens into a solid scaffold at 37°C due to temperature-induced PEG-leaching. The ability of this scaffold system to regenerate bone *in vivo* has recently been demonstrated in a mouse calvarial defect model<sup>6</sup>.

We describe the modification of this scaffold technology for potential use in mastoid air cell regeneration. A key advantage is the ability to paste the material into a cavity of any size or shape followed by scaffold solidification at body temperature. To optimise the scaffold structure we describe the inclusion of alginate beads in the formulation to act as a porogen, creating scaffolds with a high level of macroporosity. We also investigate *in vitro* release of ciprofloxacin from the scaffolds with the intention of providing local, sustained antibiotic delivery to decrease post-operative infections or continuation of underlying infections of the middle ear.

## 2. Methods

### 2.1 Mastoid bone sample preparation

A section of mastoid bone approximately 3 cm × 3 cm was excised from fresh frozen human cadaveric temporal bone.

### 2.2 PLGA/PEG particle production

Particles were fabricated from blends of poly(DL-lactic acid-co-glycolic acid) (PLGA) 85:15 DLG 4CA, 53 kDa (Lakeshore Biomaterials, USA) and poly(ethylene glycol) 400 (PEG) (Sigma Aldrich, UK) as previously described (Dhillon et al., 2011). Briefly, a mixture of 93.5%:6.5% PLGA:PEG (*w/v*) was blended at 80–90°C, mixed and allowed to

cool. Cooled polymer was ground into particles and sieved to obtain the 100–200  $\mu\text{m}$  size fraction.

### 2.3 Alginate bead fabrication

A solution of 1% ( $w/v$ ) sodium alginate (Acros organics, UK) was prepared in phosphate buffered saline. 10 ml of alginate solution was loaded into a syringe and ejected from a 23 G needle into a gently agitated bath of 25 ml 5% ( $w/v$ ) calcium chloride (Sigma-Aldrich, UK). The beads were strained using a 100  $\mu\text{m}$  sieve and rinsed with  $\text{dH}_2\text{O}$ .

### 2.4 Scaffold preparation

Triplicate scaffolds were prepared in PTFE moulds producing cylindrical scaffolds of 12 mm length and 6 mm diameter. PLGA/PEG particles were mixed with saline and varying alginate bead masses to give a range of weight percentages from 0% to 80% alginate. Ratio of PLGA/PEG particles to saline was 1:0.6 (particles:saline). Particle paste was packed into the mould which was placed at 37°C for 4 hours to allow scaffold formation. For cell seeding experiments scaffolds were UV sterilised for 80 minutes.

Ciprofloxacin-loaded scaffolds were prepared for drug release assays using two different methods, A and B. In method A, ciprofloxacin solution (Sigma-Aldrich, UK) was mixed with the PLGA/PEG particles and alginate beads to produce a paste which was used to prepare scaffolds as described above (100  $\mu\text{g}$  ciprofloxacin per scaffold). In method B, ciprofloxacin was added into the PLGA/PEG melt-blend at 80–90°C on a hotplate. The ciprofloxacin-PLGA/PEG particles were then fabricated as described in section 2.2 and used to produce scaffolds as described above (100  $\mu\text{g}$  ciprofloxacin per scaffold).

### 2.5 Porosity measurements

Triplicate scaffolds prepared using 40% PLGA/PEG-60% alginate were sintered for 4 hours at 37°C followed by freeze-drying for 24 hours causing the alginate beads to dehydrate. The porosity of the scaffolds was calculated using the bulk density and particle density values as follows:  $1 - \text{BD} / \text{PD} \times 100 = \% \text{ Porosity}$ .

### 2.6 X-ray micro-computed tomography

High-resolution three-dimensional images of the mastoid bone and scaffold samples were acquired using a laboratory X-ray micro-computed tomography system ( $\mu\text{CT}$ -40, Scanco Medical AG, Brüttisellen, Switzerland). The reconstructed field of view was 12.3mm for the scaffold specimens and 30.7mm for the mastoid bone specimen, with isotropic nominal resolutions of 12 $\mu\text{m}$  and 15 $\mu\text{m}$ , respectively. Approximately 1cm in length was scanned, spanning 800 slices (scaffolds) or 667 slices (bone). The bone or scaffold structure was binarized using a visually selected fixed global threshold. The surface of the 3D structure was triangulated and displayed using the manufacturer's software ( $\mu\text{CT}$  Ray v3.8, Scanco Medical AG).

## 2.7 Mechanical properties

PLGA/PEG particles sinter over time at 37°C temperature to form scaffolds. Assessing the mechanical properties of the scaffolds over time at 37°C reveals if solidification due to particle sintering has occurred. Triplicate scaffolds prepared using 40% PLGA/PEG-60% alginate were sintered at 37°C for 3, 4 or 5 hours. Compressive strength was tested using a TA.HD+ texture analyser (Stable Microsystems).

## 2.8 Scanning electron microscopy

Scaffolds were mounted on aluminium stubs and sputter-coated with gold at an argon current rate of 30 mA for 3 minutes. Scaffold structural morphology was examined using a scanning electron microscope (JEOL JSM-6060LV) at 10 kV.

## 2.9 Human bone marrow mesenchymal stem cell culture

Human bone marrow mesenchymal stem cells were cultured in Mesenchymal Stem Cell Media (TCS Cell Works, UK) supplemented with 5% foetal calf serum (FCS), 1% Mesenchymal stem cell growth supplement, 1% penicillin/streptomycin solution (all TCS Cell Works, UK), 1% L-Glutamine (GibcoBRL, UK) (200 mM) and 1% non-essential amino acids (Sigma-Aldrich, Poole, UK). Cells were maintained in a humidified tissue-culture incubator at 37 °C and with 5 % CO<sub>2</sub>.

## 2.10 Cell seeding on scaffolds

Triplicate scaffolds for both test and control groups were pre-wet with 100 µl media and placed in the incubator at 37°C for 1 hour.  $2 \times 10^5$  cells in a 30 µl suspension was seeded per scaffold. The scaffolds were incubated for 2 hours at 37°C prior to the addition of 1ml media to each well.

## 2.11 Prestoblue metabolic activity assay

The Prestoblue metabolic activity assay was performed on triplicate scaffolds 1, 2, 3, 5 and 7 days post-seeding. Each scaffold was submerged in 1ml of media. 111 µl of Prestoblue (Invitrogen Life Sciences, UK) was added to each well and the scaffolds were incubated at 37°C for 25 minutes. Triplicate 100 µl media samples from each well were read on a Tecan plate reader with the excitation wavelength set to 535 nm and the emission wavelength set at 615 nm.

## 2.12 Live/Dead Staining

Live/Dead staining was performed on triplicate scaffolds 3 days post-seeding. A staining solution was prepared in PBS containing 20 µg ml<sup>-1</sup> propidium iodide (Sigma-Aldrich, UK) and 1 µg ml<sup>-1</sup> fluorescein diacetate (Calbiochem, UK). 1 ml of the staining solution was added to each scaffold and incubated at room temperature for 15 minutes before visualisation using a Leica LCS confocal microscope. Live cells stain green and dead cells stain red.

### 2.13 Ciprofloxacin release assay

Triplicate scaffolds loaded with 100 µg ciprofloxacin prepared using method A or B as described in section 2.4 were placed in 3 ml PBS and incubated at 37°C. At various time intervals the PBS was replaced with 3 ml fresh PBS and 100 µl of PBS containing released drug was sampled in triplicate at 315 nm on a Tecan plate reader and concentration of drug measured. Non-drug loaded scaffolds containing only PBS were used as a control for background absorbance values. Data is presented as cumulative drug release as a function of time.

## 3. Results

### 3.1 Microstructure of human mastoid bone

Human mastoid bone microstructure was analysed using micro-computed tomography (micro-CT). The porosity of the bone was 83%. The physiology of the bone was observed in both X-ray images and 3D reconstruction (Figure 1). Pores were observed throughout the structure with sizes averaging 1.3mm, as measured by micro-CT.

### 3.2 Microstructure of PLGA/PEG-alginate composite scaffolds

Scaffolds containing different amounts of alginate beads were sintered for 4 hours. At 37°C, alginate degrades in 3–4 days whereas PLGA/PEG particles degrade in 2–3 months. Therefore the alginate beads will degrade rapidly *in vivo* leaving a highly porous PLGA/PEG scaffold structure. To mimic this degradation effect the scaffolds were freeze-dried for 24 hours prior to porosity measurements to dehydrate the alginate beads. Porosity was assessed by density measurements and increased from 43% (100% PLGA/PEG-0% alginate) to 78% (20% PLGA/PEG-80% alginate) (Figure 2A). 20% PLGA/PEG-80% alginate scaffolds were fragile when handled during cell culture experiments, therefore 40% PLGA/PEG-60% alginate scaffolds were used in all subsequent experiments (herein referred to as 'PLGA/PEG-alginate' scaffolds).

Light microscope images of PLGA/PEG-alginate scaffolds were taken before and after freeze-drying (Figure 2B). The residual pores created by the dehydrated alginate beads can be seen in the image after freeze-drying and in the micro-CT images in Figure 2C. PLGA/PEG-alginate scaffolds were submerged in saline for 2 weeks at 37°C to visually compare the physical structure of mastoid bone with the scaffolds following alginate bead degradation at this temperature (Figure 3).

### 3.3 Particle sintering within PLGA/PEG-alginate scaffolds

PLGA/PEG-alginate scaffolds were sintered for 3, 4 and 5 hours at 37°C. Compressive strength tests were performed to assess the ability of the PLGA/PEG particles to sinter in the presence of alginate beads (Figure 4). Following 3 hours at 37°C the compressive strength values were 20 kPa on average. This increased over the next hour at 37°C to 0.2 MPa and further increased to 0.38 MPa by 5 hours. The microstructure of a scaffold sintered for 4 hours at 37°C and freeze-dried for 24 hours is shown in the SEM image in Figure 4B.

### 3.4 Cell proliferation on PLGA/PEG-alginate scaffolds

Human bone marrow mesenchymal stem cells (hBM-MSCs) were seeded onto the scaffolds and the Prestoblu cell viability assay was performed over 7 days (Figure 5A). The cell number increased during this time period from  $2 \times 10^5$  cells per scaffold to  $6.8 \times 10^5$  cells per scaffold, demonstrating hBM-MSC proliferation. Scaffolds were subjected to Live/Dead staining 3 days post-cell seeding in order to visualise viable (green) and non-viable (red) hBM-MSCs within the scaffolds (Figure 5B).

### 3.5 Antibiotic release from PLGA/PEG-alginate scaffolds

Ciprofloxacin loaded scaffolds were prepared as described in Section 2.4 using two different loading methods for scaffold preparation, A and B. In method A, ciprofloxacin solution was mixed with the PLGA/PEG particles and alginate beads to produce a paste which was used to prepare scaffolds as described above (100 µg ciprofloxacin per scaffold). In method B, the appropriate amount of ciprofloxacin was added into the PLGA/PEG melt-blend. High burst release of ciprofloxacin after 24 hours (73%) was observed with the scaffolds prepared using method A (Figure 6). Burst release was minimised to 30% using method B where the drug was mixed within the PLGA/PEG at the melt-blend phase. Ciprofloxacin release from scaffolds prepared using method A slowed down to an average of 0.1% drug release per day from day 7 until day 53 when release stopped at a total of 97% ciprofloxacin released.

## 4. Discussion

An ideal material for mastoid air cell regeneration has not yet been identified, therefore alternatives have been investigated in recent years including biphasic calcium phosphate granules mixed with fibrin sealant and polycaprolactone-tricalcium phosphate composites<sup>7,8</sup>. These materials contain ceramic components which may have similar disadvantages to hydroxyapatite for this application, such as extrusion and infection. Bioactive glasses such as BonAlive have recently been reported to show promise in mastoid bone repair procedures, however the glass granules occasionally leak into the ear canal<sup>9</sup>. Biodegradable materials have also been assessed including cell-loaded hyaluronic acid gel and growth factor-loaded collagen implants<sup>10,11</sup>. These materials show promise for bone repair, however they lack the macroporosity required to regenerate the highly porous structure of the mastoid air cell system.

Here we describe the development of a biodegradable polymer scaffold tailored for mastoid air cell regeneration. The scaffold is based on PLGA/PEG particle paste which can be moulded into any size or shape and hardens into a solid scaffold at 37°C<sup>5</sup>. To our knowledge this is the first description of a biodegradable particulate polymer scaffold that can be pasted into a cavity and hardens *in vivo* for this application. The ability to paste the scaffold around the mastoid cavity offers improved clinical application and tissue approximation when compared to implants.

To aid scaffold design it is important to take into account the structure of the mastoid air cells, therefore mastoid bone was visualised using light microscopy and micro-CT. The overall porosity of the bone was measured at 83% with an average pore diameter of 1.3 mm. Our intention was to optimise scaffold structure with regard to generating large air cells

mimicking those observed in mastoid bone. Alginate beads of 1mm–1.5mm diameter were successfully used as porogens to create pores in the PLGA/PEG scaffolds. Scaffold porosity was enhanced from 43% to 78% with increasing amount of alginate beads.

In the current study, the mastoid bone sample displayed a porosity level of 83%. The scaffold formulation with a porosity value closest to that of the mastoid bone sample was 20% PLGA/PEG-80% alginate, at 78%. Due to the fact scaffolds produced using this formulation were fragile when handled during cell culture experiments, 40% PLGA/PEG-60% alginate scaffolds (64% porous) were used in all subsequent experiments. It will be important to assess the ability of both formulations to regenerate mastoid air cells in future *in vivo* studies in order to determine the best formulation to take forward for clinical use.

The ability of the scaffold to harden at body temperature is an important property as it ensures the polymer is retained where it is applied. This hardening process could potentially allow the polymer to act as an obliteration material in addition to promoting air cell regeneration within the mastoid bone. As the formulation contained 60% alginate beads it was necessary to assess the ability of the PLGA/PEG particles to sinter in this formulation. Scaffold hardening at 37°C was demonstrated by an increase in scaffold compressive strength over time, confirming the ability of the PLGA/PEG particles to fuse into a solid scaffold in the presence of the alginate beads.

PLGA/PEG scaffolds are capable of regenerating bone *in vivo*. The formulation used in the current study contained 40% PLGA/PEG and 60% alginate, therefore the ability of human bone marrow mesenchymal stem cells (hBM-MSCs) to grow on the scaffolds *in vitro* was assessed. Our results demonstrate attachment and proliferation of hBM-MSCs on the scaffolds over a seven day time period, demonstrating the potential for cells to proliferate on the scaffolds which has implications for their successful use for bone repair *in vivo*.

To reduce the likelihood of secondary complications arising from post-operative infection or recurrence of underlying middle ear infections, we incorporated ciprofloxacin into the PLGA/PEG-alginate scaffold formulation as it is commonly used in the treatment of middle ear infections. We used two different antibiotic loading methods. Method A involved mixing the drug into the PLGA/PEG and alginate bead paste prior to scaffold formation, trapping the drug inside the scaffold during solidification at 37°C. Method B involved mixing the drug into the PLGA/PEG at the melt-blend phase, therefore loading it inside the particles. Incorporation of ciprofloxacin into polymer melt-blends at high temperature (80–150°C) has been reported using polycaprolactone/poloxamine, with the antibiotic being shown to retain its antibacterial activity following exposure to these high temperatures<sup>12</sup>. In our method the ciprofloxacin was heated to 80°C. A high burst release of 73% ciprofloxacin after 24 hours was observed with the scaffolds prepared using method A, caused by drug on the surface of the particles releasing immediately. This burst release was minimised to 30% using method B, as the drug is trapped inside the particles therefore reducing the amount of drug available for immediate release. Scaffolds produced using method A released for 7 weeks, with a daily release rate of approximately 0.1% per day. Scaffolds produced using method B



generated an improved release profile with a lower burst release phase followed by 10 weeks release of approximately 1% per day.

## 5. Conclusions

We describe the development of a porous, biodegradable PLGA/PEG-alginate scaffold that can be pasted into a cavity of any size or shape. Scaffold porosity can be controlled by varying the amount of alginate beads in the formulation, with a maximum of porosity of 78% achieved. The scaffolds support the growth of hBM-MSCs and display sustained release of ciprofloxacin for 7–10 weeks *in vitro*. Future studies will focus on the assessment of this scaffold formulation for mastoid air cell regeneration *in vivo*.

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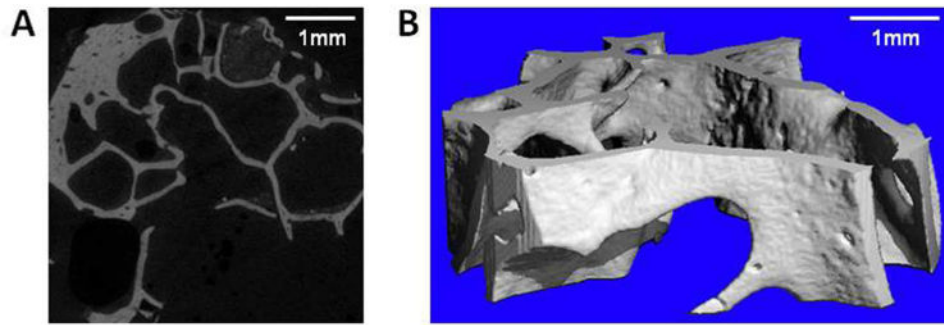
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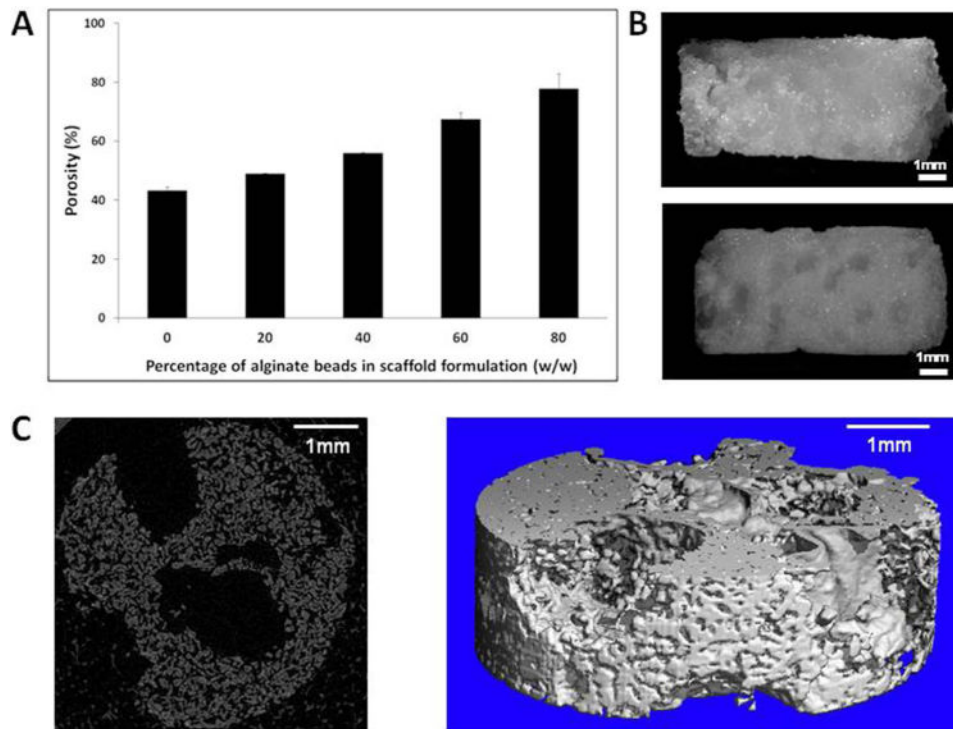
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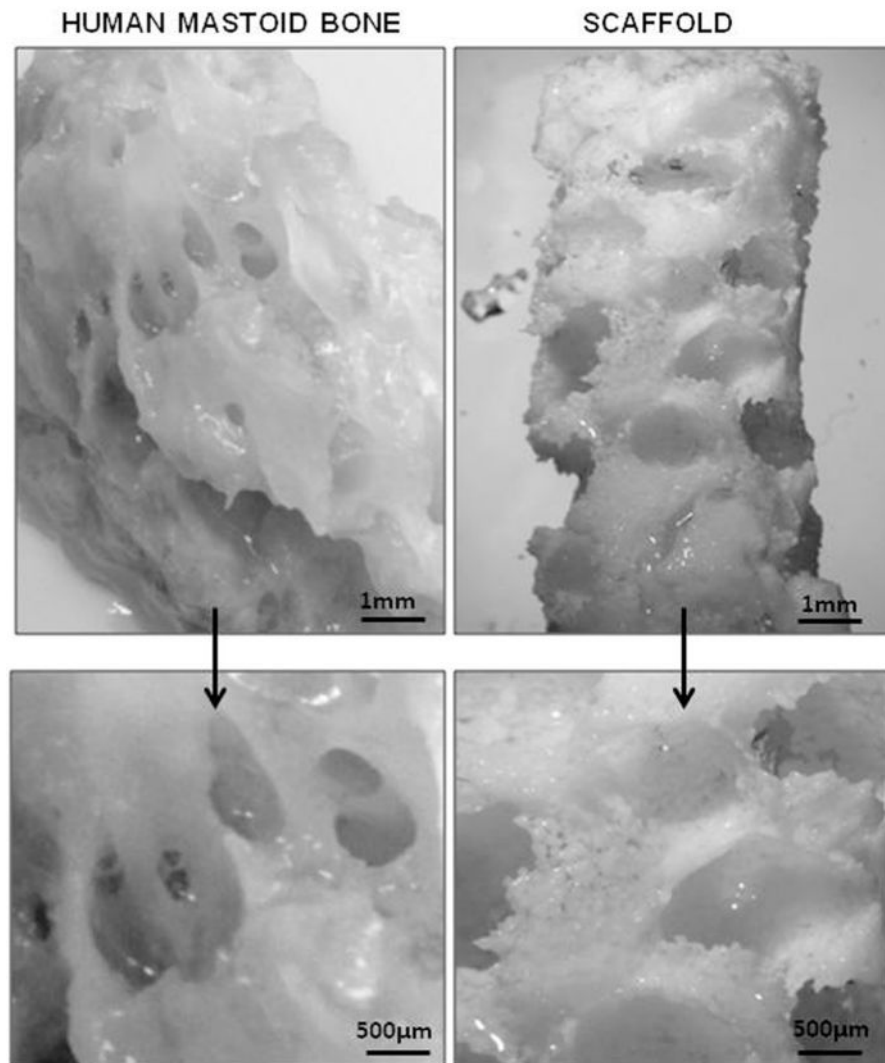
**Figure 1. Microstructure of human mastoid bone**

(A) Micro-CT x-ray slice image of human mastoid bone. (B) Micro-CT 3D reconstruction of human mastoid bone.

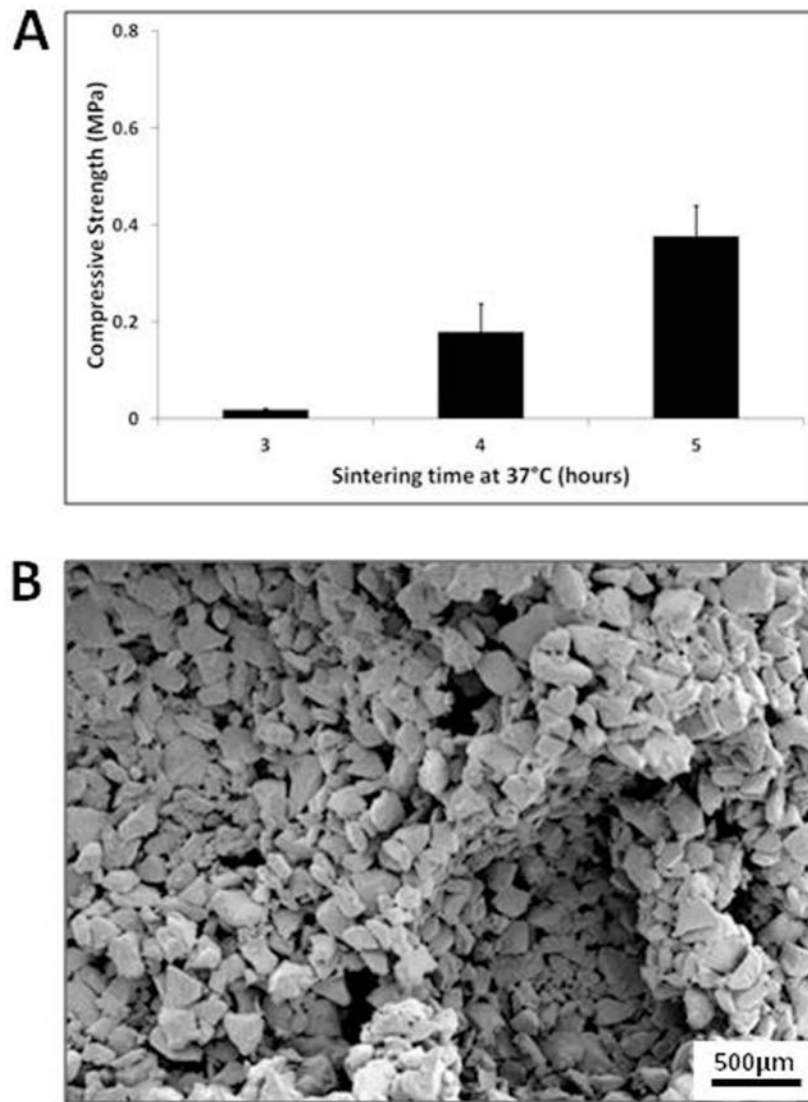


**Figure 2. Microstructure of PLGA/PEG-alginate scaffolds**

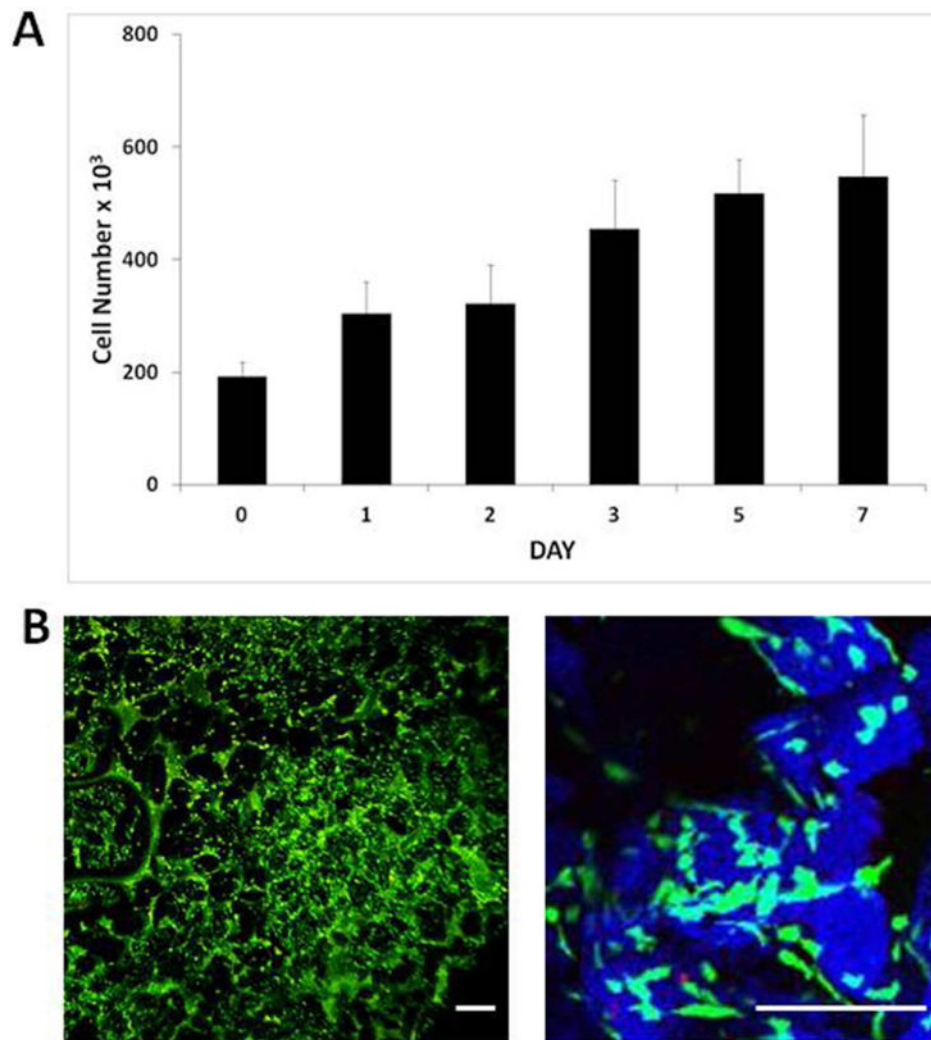
(A) Porosity of PLGA/PEG-alginate scaffolds calculated by density measurements. (B) Light microscope images of 40% PLGA/PEG-60% alginate scaffolds before freeze-drying (top image) and after freeze-drying for 24 hours (bottom image). (C) Micro-CT x-ray slice and 3D reconstruction of 40% PLGA/PEG-60% alginate scaffold after freeze-drying for 24 hours.



**Figure 3. Image comparison of human mastoid bone and PLGA/PEG scaffold**  
Light microscope image of human mastoid bone section (left) and 40% PLGA/PEG-60% alginate scaffold (right). Scaffold was submerged in saline at 37°C for 2 weeks prior to imaging.

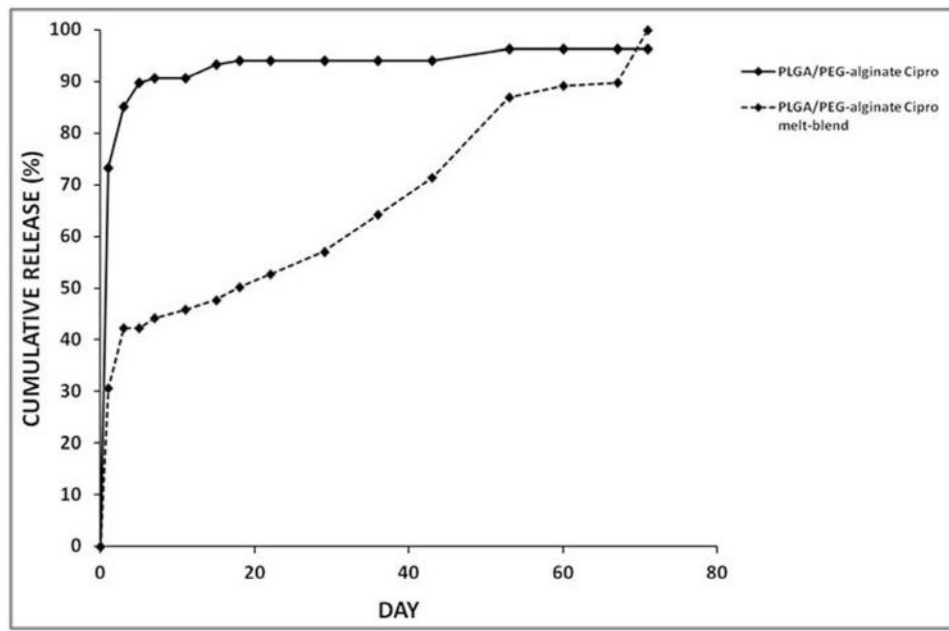


**Figure 4. PLGA/PEG particle sintering within PLGA/PEG-alginate scaffolds at 37C**  
(A) Compressive strength of 40% PLGA/PEG-60% alginate scaffolds sintered for 3, 4 and 5 hours at 37°C. Error bars represent standard deviation. (B) Representative scanning electron microscopy image of a 40% PLGA/PEG-60% alginate scaffold sintered for 4 hours at 37°C and freeze-dried for 24 hours.



**Figure 5. Proliferation of human bone marrow-derived mesenchymal stem cells on PLGA/PEG-alginate scaffolds**

(A) Number of viable human bone marrow mesenchymal stem cells (hBM-MSCs) on 40% PLGA/PEG-60% alginate as measured by the Prestoblu metabolic activity assay over 7 days in culture. Error bars represent standard deviation. (B) Representative images of hBM-MSC-seeded 40% PLGA/PEG-60% alginate scaffold 3 days post-seeding following Live/Dead staining. PLGA/PEG particles rendered in blue using Leica LCS confocal microscope software in order to visualise the particles (blue) and pore spaces between particles (black) Size bar = 200 $\mu$ m.



**Figure 6. Ciprofloxacin release from PLGA/PEG-alginate scaffolds**

Cumulative release profiles for ciprofloxacin from 40% PLGA/PEG-60% alginate scaffolds represented as percentage of total loaded drug released. Two different drug loading methods were used, method A ('PLGA/PEG-alginate cipro') and method B (PLGA/PEG-alginate cipro melt-blend').