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Investigation of localised delivery of diclofenac sodium from PLGA/PEG scaffolds using an in vitro osteoblast inflammation model.

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	comprised of mouse primary calvarial osteoblasts stimulated with proinflammatory cytokines interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Levels of inflammation were monitored by cell viability and cellular production of nitric oxide (NO) and prostaglandin E_2 (PGE_2). The osteoblast inflammation model revealed that proinflammatory cytokine addition to the medium reduced cell viability to 33%, but the release of diclofenac sodium from scaffolds inhibited this effect with a final cell viability of approximately 70%. However, releasing diclofenac sodium at high concentrations had a toxic effect on the cells. Proinflammatory cytokine addition led to increased NO and PGE_2 production; diclofenac sodium releasing scaffolds inhibited NO release by approximately 64% and PGE_2 production by approximately 52%, when the scaffold was loaded with the optimal concentration of drug. These observations demonstrate the potential use of PLGA/PEG scaffolds for localised delivery of anti-inflammatory drugs in bone tissue engineering applications.

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Abstract

Non-union fractures and large bone defects are significant targets for osteochondral tissue engineering strategies. A major hurdle in the use of these therapies is the foreign body response of the host. Herein, we report the development of a bone tissue engineering scaffold with the ability to release anti-inflammatory drugs, in the hope of evading this response. Porous, sintered scaffolds composed of poly(DL-lactic acid-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) were prepared with and without the anti-inflammatory drug diclofenac sodium. Analysis of drug release over time demonstrated a profile suitable for the treatment of acute inflammation with approximately 80% of drug released over the first 4 days and a subsequent release of around 0.2% per day. Effect of drug release was monitored using an *in vitro* osteoblast inflammation model, comprised of mouse primary calvarial osteoblasts stimulated with proinflammatory cytokines interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Levels of inflammation were monitored by cell viability and cellular production of nitric oxide (NO) and prostaglandin E_2 (PGE_2). The osteoblast inflammation model revealed that proinflammatory cytokine addition to the medium reduced cell viability to 33%, but the release of diclofenac sodium from scaffolds inhibited this effect with a final cell viability of approximately 70%. However, releasing diclofenac sodium at high concentrations had a toxic effect on the cells. Proinflammatory cytokine addition led to increased NO and PGE_2 production; diclofenac sodium releasing scaffolds inhibited NO release by approximately 64% and PGE_2 production by approximately 52%, when the scaffold was loaded with the optimal concentration of drug. These observations demonstrate the potential use of PLGA/PEG scaffolds for localised delivery of anti-inflammatory drugs in bone tissue engineering applications.

Introduction

Non-union fractures, large bone defects and diseases such as rheumatoid arthritis are significant targets for osteochondral tissue engineering strategies. Under normal circumstances, bone has a significant capacity for regeneration and is one of the few tissues that can heal without a scar (1). Fracture healing consists of three stages: inflammation; repair; and remodelling. The initial inflammatory phase plays a critical role in healing and many of the processes that occur at this stage determine the outcome of bone repair. Within 24 hours of bone injury, neutrophils and macrophages will have migrated into the wound site and levels of tumour necrosis factor α (TNF-α), interleukin 1 (IL-1), interferon-γ (IFN-γ) and interleukin 6 (IL-6) will reach their peak (2-4). These cytokines lead to recruitment of additional cells involved in inflammation, promotion of angiogenesis and mesenchymal stem cell (MSC) differentiation. The acute inflammatory phase usually lasts for one week, after which levels of inflammatory mediators return to baseline (4-7). During the subsequent remodelling phase of fracture healing, the expression levels of cytokines such as IL-1, TNF-α and IL-6 rise once more, but do not reach the levels of the acute inflammatory response (8).

Due to the complexity of processes involved in fracture healing, there are clinical indications where inflammation fails to resolve the problem, such as inflammatory diseases, severe body reactions and non-union fractures. The current “gold-standard” treatment for non-union fractures and defects is autologous bone grafts. However, such grafts are limited due to availability and have disadvantages such as donor site morbidity, additional surgery and chronic pain. This has inspired the development of tissue engineered bone substitutes. These bone substitutes are required to provide mechanical stability, support cell growth, and aid bone

regeneration. A common disadvantage of any tissue-engineered therapy is inflammation and foreign body response, upon implantation and degradation (9). Thus, the adaptation of a bone tissue engineering scaffold that provides release of anti-inflammatory mediators could potentially enhance the properties of the therapy and improve success rates upon implantation (10, 11).

In this study, we investigate release of the anti-inflammatory drug diclofenac sodium from porous poly(DL-lactic acid-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG) microparticle-based scaffolds. Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, have systemic treatment side-effects of gastrointestinal ulceration and bleeding, hepato-renal dysfunction and skin reactions (12). Local delivery of this drug via scaffolds offers a method of bypassing the side effects of systemic drug treatment. The properties of the PLGA/PEG scaffolds have been reported and are established as suitable for bone repair (13-15). The scaffold has demonstrated efficacy in the sustained release of growth factors, antibiotics and chemotherapeutics (14, 16, 17). The potential exploitation of the scaffold as an anti-inflammatory drug delivery vehicle will enhance the existing osteoinductive properties of the PLGA/PEG scaffolds in bone repair. The scaffolds are manufactured from PLGA/PEG microparticles that form a paste when mixed with a carrier solution. This paste can be easily injected or pasted into the area of interest and subsequently solidifies at body temperature forming a porous scaffold.

Current validation methods for testing the properties and effectiveness of anti-inflammatory drugs released as part of tissue engineering studies are predominantly *in vivo* animal models (18-20). Current *in vitro* models are few and far between but involve the use of tissue slices (21-23). In this study, a simple *in vitro* calvarial osteoblast inflammation model is created using the proinflammatory cytokines IL-1β,

TNF-α and IFN-γ. This model is then used to evaluate the effects of anti-inflammatory drug release from the PLGA/PEG scaffolds. Common markers of the effects of proinflammatory cytokines on osteoblasts, such as viability, nitric oxide (NO) production and prostaglandin E_2 (PGE_2) production are used to assess the efficacy of drug release (24, 25). The *in vitro* model is not intended to replace animal models, but offers a simple initial step to gather information about effectiveness of drug release from scaffolds.

Materials and Methods

Extraction and culture of primary calvarial osteoblasts

Primary calvarial osteoblasts were obtained from 1-3 day old CD1 mouse calvaria by sequential enzymatic digestion. Calvaria were dissected and digested in a solution of 1.4 mg/mL collagenase type IA (Sigma Aldrich, Poole, UK) and 0.5 mg/mL trypsin II S (Sigma). Cells released during the first 2 digestions (10 min) were discarded, and populations of cells from the next 3 digestions (20 min) were plated in tissue culture flasks at a density of 6.6 x 10^3 cells/cm^2. Cells were cultured in minimum essential medium-α (αMEM, Lonza, Belgium), containing 10% FBS (Sigma), 2 mM L-Glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma). For experimentation, cells were passaged via trypsin/EDTA, plated at a density of 10,600 cells/cm^2 and allowed to adhere overnight in well plates. Medium was subsequently supplemented with 50 mM β-Glycerophosphate (BGP, Sigma) and 50 µg/mL ascorbate 2-phosphate (Sigma), to induce osteogenic differentiation.

Proinflammatory Cytokine Treatment

Recombinant human IL-1β, human TNF-α and mouse IFN-γ (R&D Systems), were added to the culture medium at a concentration of 0.25 ng/mL, 2.5 ng/mL and 25 ng/mL respectively, unless otherwise stated.

MTS Viability Assay

A tetrazolium salt assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, UK, CellTiter 96® AQueous One Solution Cell Proliferation Assay) was performed as per the manufacturer's protocol. Briefly, 20 µL MTS solution was added to 100 µL culture media. After incubation for 1 hr at 37°C, the absorbance of each well was measured at 490 nm using plate reader.

Nitric Oxide and Prostaglandin E_2 Production

Nitrite in culture medium was measured as an estimation of NO production, using the Griess Reagent System (Promega). Nitrite concentration within culture medium samples was determined by adding 1% sulphanilamide in 5% phosphoric acid, incubating for 5 minutes before adding 0.1% N-(1 napthyl)ethylenediaminedihydrochloride (NED) in water. Absorbance was read after 5 minutes room temperature incubation at 540 nm with wavelength correction at 690 nm.

Prostaglandin E_2 (PGE_2) concentration within culture medium was determined by enzyme immunoassay (EIA) using a commercially available kit (R&D Systems). Briefly, a competitive binding EIA was performed on 3-fold diluted samples. Optical absorbance was read at 450 nm with correction at 570 nm.

Immunocytochemistry

After treatment with diclofenac sodium and/or proinflammatory cytokines, cell monolayers were fixed with a 4% solution of paraformaldehyde (Sigma). Cells were permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 45 minutes and washed with PBS. Nonspecific binding was blocked by incubating in 3% donkey serum (Sigma) for 30 minutes. Samples were incubated with primary antibodies at 4°C overnight. PBS washes were performed and samples incubated with secondary antibodies (donkey Alexa-Fluor-488 or 546, Invitrogen) for 1 hour at room temperature. Nuclear counterstaining with Hoechst 33258 was performed. Primary antibodies were goat anti-osteopontin (R&D Systems), goat anti-osteocalcin (Millipore, UK), rabbit anti-collagen-I (Millipore, UK) and goat anti-cadherin-1 (R&D Systems). Fluorescent images were captured and processed using a Leica DM-IRB inverted microscope and Volocity imaging software (Improvision, Coventry, UK).

PLGA/PEG Particle Production

Thermosensitive particles were manufactured from 53kDa P_{DL}LGA (85:15 DLG 4CA) (Evonik Industries, USA) and PEG 400 (Sigma Aldrich, UK) as previously described (13). Briefly, the PLGA and PEG were blended at a ratio of 93.5:6.5 (w/v), at 80-90°C

on a hotplate and allowed to cool. Polymer blend sheets were ground into particles in a bench-top mill (Krups Mill F203) and sieved to obtain a 100-200 μm particle size fraction.

Diclofenac Sodium Loaded PLGA/PEG Scaffold Manufacture

Scaffolds were prepared in PTFE moulds producing cylindrical scaffolds of 12 mm length and 6 mm diameter. PLGA/PEG particles were mixed manually at a ratio of 1:0.6, particles to PBS carrier solution. Diclofenac sodium (Sigma) loaded scaffolds were produced by mixing particles with PBS containing diclofenac sodium. The microparticle/PBS paste was packed into the moulds. Solid scaffolds were produced after sintering at 37°C for 3 hours.

Scanning Electron Microscopy

Scaffolds were cut in half to allow the centre to be imaged. Cut scaffolds were mounted on aluminium stubs and were sputter-coated with gold at an argon current rate of 30 mA for 3 min. The structural morphology of the scaffolds was examined using a SEM (JEOL JSM-6060LV) at 10 kV.

Measurement of drug release from PLGA/PEG Scaffolds

After sintering, scaffolds were removed from moulds and placed into phenol red-free αMEM (Invitrogen, UK), with 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37°C. Release medium was removed for drug concentration

measurements, and fresh release medium added. Drug release was measured by ultraviolet-visible (UV-Vis) spectrophotometry at 276 nm. Concentrations of drug within the medium were determined using calibration curves.

Calvarial osteoblast inflammation model

Osteoblasts were cultured in monolayer for 14 days in osteogenic medium. UV-sterilised scaffolds were placed in transwells above the cell monolayer with medium added to cover scaffold. During diclofenac-release experiments, medium was changed to phenol-red free αMEM containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM L-Glutamine, 50 mM BGP and 50 µg/mL ascorbate 2-phosphate. In experimental groups stimulated with cytokines, initial concentration was 1 ng/mL IL-1β, 10 ng/mL TNF-α and 100 ng/mL IFN-γ. Medium was collected after 24 hours and replaced with medium containing 0.25 ng/mL IL-1β, 2.5 ng/mL TNF-α and 2.5 ng/mL IFN-γ. This medium was collected after a further 24 hours and replaced with 0.0625 ng/mL IL-1β, 0.625 ng/mL TNF-α and 6.25 ng/mL IFN-γ. This medium was left for a further 5 days until experiment end at day 7.

Live/Dead™ Viability Assay

Live/Dead™ viability/cytotoxicity assay (Invitrogen) was performed on the cell monolayers at the end of scaffold release experiments (day 7). Cell monolayers were incubated with a solution of 2 µM calcein AM and 4 µM EthD-1 in PBS for 30 minutes at room temperature. Monolayers were imaged using an inverted fluorescent

microscope. Live/dead quantification was performed using ImageJ version 1.43U (NIH, USA).

Statistical Analysis

Statistical significance between groups was analyzed using PASW Statistics 18.0.3 software. Two groups were compared by unpaired Student's T-Test, multiple groups by one-way ANOVA.

Results

Effect of diclofenac sodium and IL-1β, TNF-α and IFN-γ on the *in vitro* responses of primary osteoblasts

The effects of diclofenac sodium on proinflammatory cytokine treated osteoblasts were assessed prior to release from scaffolds. Primary osteoblasts were cultured in osteogenic medium for 14 days before cytokines and diclofenac added. Cell viability was monitored over the following 21 days (figure 1A). Adding diclofenac sodium caused a reduction in viability over the 21 days, showing slight toxicity to the cells. The addition of IL-1β, TNF-α and IFN-γ caused a significant drop in viability over the 21 day period, to 32% (±14%) compared with the control. The addition of diclofenac with cytokines inhibited the cytokine-induced cell death, with a viability of 92% (±11%) at day 14 and 62% (±2.5%) at day 21.

Figures 1B and 1C show cumulative production of nitrite and PGE_2 respectively, by primary osteoblasts, in the presence of diclofenac sodium and/or proinflammatory

cytokines. Addition of IL-1β, TNF-α and IFN-γ to the medium caused significantly increased nitrite and PGE_2 production, compared to control osteogenic medium and 100 μM diclofenac. Nitrite accumulation in groups treated with both cytokines and diclofenac was significantly lower across all timepoints than those with cytokines alone. However, nitrite levels in this group remained significantly higher than the controls. Diclofenac sodium was effective in inhibiting PGE_2 production. Accumulated PGE_2 concentration in the group treated with cytokines and diclofenac remained similar to controls across all timepoints. Diclofenac sodium was shown to maintain effectiveness as an NSAID in this model across a 27-day time period.

Figure 1D and 1E show the effect of the cytokines and diclofenac on primary osteoblast differentiation, with staining for osteopontin (OPN) with osteocalcin (OCN) (1D) and collagen-I with cadherin-11 (1E). Osteoblasts were cultured for 14 days in osteogenic medium before the addition of cytokines and diclofenac. Staining was subsequently performed on day 21. Compared to osteogenic medium, treatment with proinflammatory cytokines produced smaller and less abundant nodules with dense OPN staining, little OCN and more disperse collagen-I staining. Hoechst staining showed that there were fewer cells within nodules relative to osteogenic controls. In cells treated with proinflammatory cytokines and diclofenac, the nodules were of a similar size and cell density to the controls and denser staining for osteocalcin, collagen-1 and cadherin-11 was seen than in those treated with cytokines alone.

Diclofenac sodium release from PLGA/PEG scaffolds

The PLGA/PEG scaffolds had a porous microstructure (figure 2A) formed by particles fusing together at 37°C. When unconstrained, scaffolds showed a small

amount of swelling after being placed in medium for drug release (figure 2B). In later studies, with release onto cell monolayers, scaffolds were constrained by transwells and release remained unchanged (data not shown).

The release of diclofenac sodium from the PLGA/PEG scaffolds over time was assessed by UV spectrophotometry. The cumulative mass drug release can be seen in figure 3A, and converted to percentage of initial loading in figure 3B. Scaffolds were loaded with different concentrations of diclofenac to determine effect of initial drug loading on release profile. All scaffolds showed a similar release profile regardless of original loading, with an initial burst release phase of 55-62% over the first 4 hours, followed by approximately 20% release by day 1, decreasing to 3% release by day 2. After day 4, release reached a steady state of around 0.2% per day. From day 42, this rate increased to about 0.3% per day as the scaffolds started to degrade. By day 68, the scaffolds had degraded and average of 96% (±3%) of the loaded drug had been released per scaffold.

Viability of primary calvarial osteoblasts after diclofenac sodium release from PLGA/PEG scaffolds

Diclofenac releasing scaffolds were placed in transwells above osteoblast monolayers and culture medium with and without IL-1β, TNF-α and IFN-γ added. Live/dead images were taken of the primary osteoblast monolayers after 7 days drug release from the scaffolds (figure 4A) and images quantified (figure 4B and 4C). In osteogenic medium with no scaffold and 0 μg, 300 μg and 650 μg drug-loaded scaffolds, there were very few dead cells. An initial load of 1000 μg caused a large increase in the number of dead cells and lowered the percentage viability to below

20%, indicating a toxic burst-release. In medium containing IL-1β, TNF-α and IFN-γ, with no scaffold and 0 µg scaffolds, there was a large fall in cell viability, to 33% and 23% respectively. Release of diclofenac from the 300 µg and 650 µg scaffolds improved cell viability in cytokine medium to 76% and 68% correspondingly. The 1000 µg loaded scaffold in cytokine medium showed an enhanced negative effect with significantly less total cells and a viability of 3%.

Effect of diclofenac sodium-releasing PLGA/PEG scaffolds on NO production in the osteoblast inflammation model

Levels of nitrite in the medium were measured after day 1, day 2 and at day 7, to estimate NO production (figure 5). In osteogenic control medium with and without scaffolds, there was minimal NO production. When proinflammatory cytokines were present, the control (no scaffold) and the 0 µg scaffold showed significantly increased NO, at all timepoints. The 300 µg diclofenac releasing scaffolds showed no significant NO production on day 1, but day 2 and day 7 levels were significantly increased. The 650 µg scaffolds showed inhibition of NO production at all timepoints, although by day 7, concentrations were also increased significantly higher than no cytokine control. Scaffolds releasing 1000 µg diclofenac, showed low nitrite production across all timepoints, but low levels can be correlated with low cell viability. Cumulative nitrite production across the 7 days (figure 5B) shows that the 650 µg loaded scaffolds significantly inhibited cytokine-induced NO production, with a reduction of approximately 64%, compared with the 0 µg scaffold in cytokine medium.

Effect of diclofenac sodium-releasing PLGA/PEG scaffolds on PGE_2 production in the osteoblast inflammation model

Concentration of PGE_2 in the culture medium was measured on day 1, day 2 and day 7 (figure 6). PGE_2 concentration across all groups, at all timepoints can be seen in figure 6A. When all groups are evaluated on the same graph, the "no scaffold" experimental group in cytokine medium shows a very large amount of PGE_2 production, skewing the data; this can also be seen in the cumulative data (figure 6B). It was expected that similar levels of PGE_2 would be produced by cells with 0 µg scaffolds, but this was not observed when assaying the medium. This effect could be attributed to either some of the PGE_2 or proinflammatory cytokines adsorbing to the scaffold and therefore not remaining soluble in the medium, causing variation in results between those with the scaffold present and those without. However, figure 6A does show a trend of increased PGE_2 in the 0 µg scaffold on day 1 and day 2, compared to the 300 µg, 650 µg and 1000 µg diclofenac-releasing scaffolds, indicating some inhibition of cytokine-induced PGE_2 by the presence of diclofenac. To get a more valuable understanding of how the scaffolds are affecting PGE_2 response, cumulative results excluding the "no scaffold" controls can be found in the inset of figure 6B. This shows that at all timepoints, the 0 µg scaffold showed increased PGE_2 production in proinflammatory cytokine medium compared to control osteogenic medium. Cumulative PGE_2 concentration in the medium until day 7 showed that all diclofenac sodium loaded scaffolds, showed significant inhibition of cytokine-induced PGE_2 production, with a reduction of 51.9% compared to the 0 µg scaffold in cytokine medium.

Discussion

A major hurdle in the use of tissue-engineered therapies is the foreign body reaction of the host, associated with the implantation of the therapy. The aim of this study was the examination of PLGA/PEG scaffolds as a release vehicle for anti-inflammatory drugs, with the eventual aim of evading the foreign body response *in vivo*. Diclofenac sodium was chosen as the drug of choice to test this system for several reasons: NSAIDs show appealing properties as an anti-inflammatory in bone treatment; good solubility in water; stability over time; successful results in both PGE_2 and nitrite inhibition during *in vitro* tests; and finally, available literature describing release of diclofenac via various techniques (26-28).

PGE_2 and NO are important inflammatory mediators produced by osteoblasts in response to proinflammatory cytokines, and can be used to monitor levels of inflammation (24, 25, 29). The major mode of action of NSAIDs, such as diclofenac, is inhibition of PGE_2 production through the cyclooxygenase-2 (COX-2) pathway (30, 31). COX-2 is an inducible enzyme, generated in response to proinflammatory cytokine signalling, particularly IL-1 and TNF-α, leading to significantly increased production of PGE_2 (32). The production of prostaglandins, such as PGE_2, leads to the initial recruitment of neutrophils and subsequent recruitment of macrophages (33). The release of diclofenac from a scaffold would potentially lead to less neutrophils and macrophages at the injury site, decreasing the acute inflammatory response that leads to pain, swelling and rejection. In some tissues this would lead to poor regeneration, however in bone, an absence of neutrophils and macrophages may not adversely affect tissue repair (34, 35). Macrophages have different phenotypes depending on the local tissue environment; the classically activated M1 phenotype is present during acute inflammation and is linked to upregulation of

proinflammatory cytokines and enzymes such as inducible nitric oxide synthase (iNOS). During tissue regeneration, macrophages transition to an anti-inflammatory M2 phenotype that plays a role in dampening inflammation by the production of anti-inflammatory cytokines such as IL-10 and IL-1ra (36, 37). The COX-2 pathway has been linked with the transition of M1 macrophages into M2 phenotypes, and thus treatment with diclofenac may cause a delay in the transition of M1 macrophages (38, 39). However in this system, effective anti-inflammatory release would only occur during the initial inflammatory stage and this transient exposure may not be enough to delay subsequent regeneration.

In this study, diclofenac sodium successfully inhibited all cytokine-induced production of PGE_2 in initial long-term cell monolayer tests. Inhibition of PGE_2 production was also seen when diclofenac was released from scaffolds, although results were hampered by potential adsorption of PGE_2 to the scaffold. Despite the success in this model, the complete inhibition of PGE_2, and therefore COX-2, by an NSAID may not be completely desirable, as COX-2 plays a role in bone regeneration and fracture healing (40-42). In consideration of this, the drug diclofenac may not be optimal for this purpose but in future the system could be adapted for release of anti-inflammatory drugs with alternative mechanisms of action. It is crucial to maintain the correct balance between effective anti-inflammatory function to prevent foreign body reaction, and the ability of the scaffold to regenerate bone, for this system to be successful.

The ability of NSAIDs to inhibit NO production is not as well recognized, but did occur to a certain degree in this study, in initial long-term studies and during diclofenac release from scaffolds. This result is not unexpected as NSAIDs have been shown to inhibit iNOS activity, which leads to inflammation induced NO

production (43, 44). This process does not cause complete inhibition of NO production, and unlike the mode of action of anti-inflammatory glucocorticoids, is not caused by inhibition of iNOS mRNA (45). The diclofenac sodium results in this study show partial inhibition of NO production, in agreement with this statement. Interactions have previously been shown to exist between the COX and iNOS pathways in a number of cells, including osteoblasts (46-49); however the inhibitory effects of NSAIDs on NO production have been shown to be both COX-dependent and COX-independent (44, 48). Development and validation of the simple osteoblast inflammation model, led to the development of a tissue engineered drug release system. The injectable PLGA/PEG scaffold formulation has previously been reported and its suitability for bone tissue engineering has been demonstrated (13-15). The scaffolds were prepared from PLGA/PEG particles that form a paste when mixed with a carrier solution, in this case containing diclofenac. The paste hardens into a solid, porous scaffold when sintered at 37°C, due to temperature-induced PEG-leaching (13). During the sintering process the diclofenac sodium in the carrier, most likely becomes adsorbed or absorbed within the particles and therefore becomes physically entrapped within the pores of the resulting scaffold structure. Drug release in this system is uncontrolled, occurring via diffusion through the pores; release therefore depends upon drug properties and interaction between the drug and polymer. In this study, release consisted of a large burst within the first few hours due to drug molecules adsorbed to the surface of the scaffold immediately releasing into the medium. In some cases, this would be considered an undesirable effect, but in the case of *in vivo* inflammation, this burst release of anti-inflammatory drug correlates with an influx of proinflammatory cytokines upon implantation of the scaffold. After the burst, drug release slowed rapidly. From day 1 to day 4, release

can be explained by diffusion of drug through water-filled pores. From day 4, release slowed substantially, but followed an almost first-order release profile, as the remaining drug found a way through the scaffold, diffusing into the medium.

Initial loading of the drug within the scaffold had little effect on the release profile and consistency of release between batches of scaffolds correlated well. The length of time over which the majority of the diclofenac was released was about 6 days. This is in keeping with other tissue engineered anti-inflammatory release strategies that have shown 3-6 days (27, 50, 51). Longer more controlled release could be achieved by using other polymer systems, such as encapsulating the drug within microspheres fabricated by the emulsion technique (52, 53). The PLGA/PEG system described herein could also be adapted by incorporating the drug into the melt-blend of the PLGA/PEG as previously demonstrated, to alter the release profile (16).

Diclofenac release from scaffolds onto the osteoblast inflammation model was performed using a transwell system. This allowed the cell monolayer to be kept separate from the scaffold and did not introduce levels of complexity allowing migration of cells onto the scaffold. The transwell system allowed drug release into the cell culture medium which could freely diffuse through the pores of the membrane to the cell monolayer. The system had disadvantages; the scaffold swelled as water was imbibed and became constrained by the edges of the transwell, possibly affecting release. Nevertheless, as experiments were kept to a maximum of 7 days, this effect had little time to come into consequence. Overall, the simple system worked successfully to demonstrate that diclofenac sodium release from the scaffold remained effective as an anti-inflammatory. An issue that emerged as the system developed was adsorption of PGE_2 and medium proteins, such as the proinflammatory cytokines, to the PLGA/PEG scaffold. This was reflected most

obviously in results for PGE_2, but may also have been seen in nitrite readings, resulting in lower values when scaffolds were present. However, although there were differences between groups with and without scaffolds, when comparing scaffolds that released diclofenac to scaffolds that were not loaded with any drug, there were still significant differences, showing inhibition of the actions of the proinflammatory cytokines.

Effective drug concentration was indicated by cell viability within the osteoblast inflammation model. This was important, as anti-inflammatory drugs have been shown to induce apoptosis and arrest the cell cycle (54, 55). Achieving a balance between mass of drug released to attain effective anti-inflammatory dose and the maintenance of cell viability is difficult. The highest scaffold loading of 1000 µg/scaffold showed almost complete cell death by day 7 of release, a property of the large initial burst. This large cell death rendered results of PGE_2 and nitrite obsolete, as low values only indicated low cell numbers. There was more success with scaffolds loaded with 300 µg or 650 µg diclofenac sodium. Results indicate that an initial loading between the two would give optimum results for cell viability and inhibition of inflammatory markers.

The *in vivo* inflammatory environment is vastly different to the one created in this study but as an initial validation system, prior to *in vivo* studies, it was successful. Overall, the PLGA/PEG scaffolds released the diclofenac in a profile that would be effective for treatment of acute inflammation and the *in vitro* inflammation model demonstrated that diclofenac sodium was effective after release.

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Author Disclosure Statement

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Figure Legends

Figure 1: Long term effect of diclofenac sodium on response of primary calvarial osteoblasts in proinflammatory cytokine medium. Osteoblasts were cultured for 14 days in osteogenic medium before experimentation. Cytokines (0.25 ng/mL IL-1β, 2.5 ng/mL TNF-α, 25 ng/mL IFN-γ) were applied throughout the 27 day period. (A) Cell viability, converted to percentage of control medium reading. Values represented as mean±SD, n=6. Experiment repeated in triplicate. (B) Cumulative nitrite concentration in culture medium at timepoints. Values represented as cumulative mean±cumulative SD, n=6. Experiment repeated in triplicate. (C) PGE_2 concentration in medium at timepoints. Values represented as cumulative mean±cumulative SD. Experiment repeated in triplicate. For A, B, C: Statistical significance vs. control *(p≤0.01). Statistical significance vs. IL-1β+TNF-α+IFN-γ #(p≤0.01). (D) Representative immunocytochemistry images showing expression of osteopontin (OPN)/osteocalcin (OCN) and cadherin-11 (cad-11)/collagen-I (col-I) after treatment with IL-1β, TNF-α and IFN-γ and diclofenac sodium. Scale bar=90 μm.

Figure 2: PLGA/PEG scaffolds. (A) Representative SEM images of scaffold microstructure. (B) Formed and sintered scaffolds showing swelling and degradation over 5 weeks.

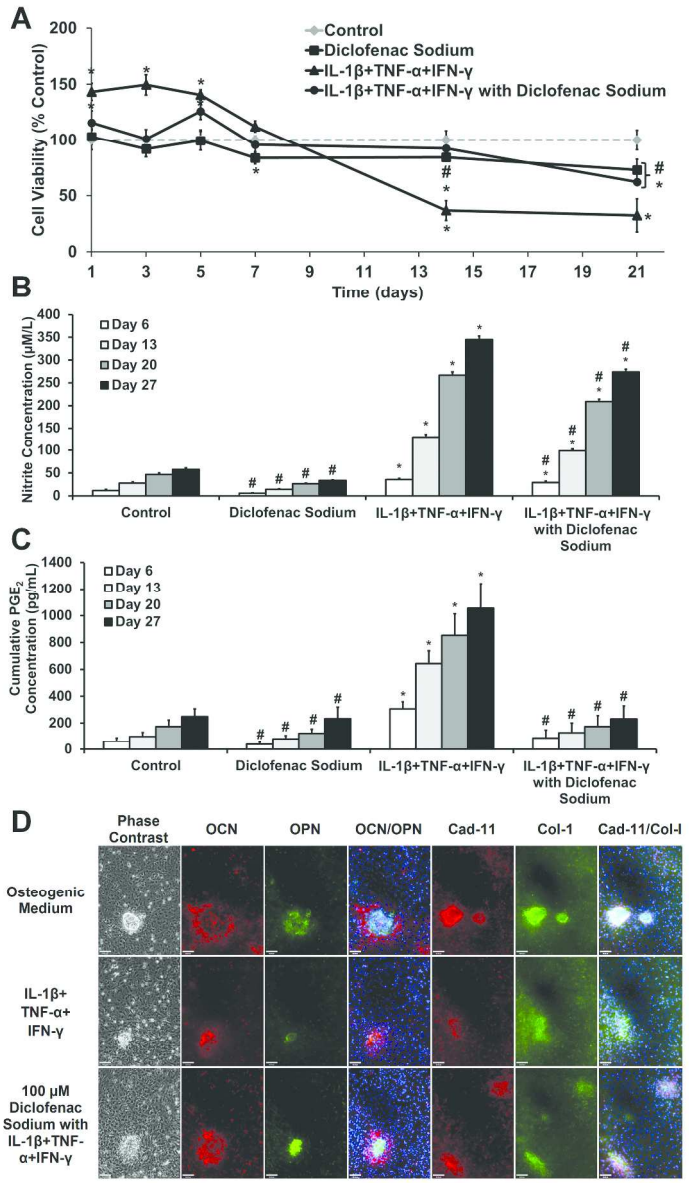
Figure 3: Diclofenac sodium release from PLGA/PEG scaffolds. Scaffolds were loaded with varying initial concentrations of diclofenac sodium. (A) Cumulative mass release of drug over time. (B) Cumulative percentage release of drug over time. Values represented by mean of triplicate scaffold release, each measured in triplicate, n=9. Error represented by cumulative SD.

Figure 4: Effect of diclofenac sodium releasing PLGA/PEG scaffolds on cell viability in the *in vitro* calvarial osteoblast inflammation model. Live/Dead™ viability assay of osteoblast monolayers after 7 days culture with and without IL-1β, TNF-α and IFN-γ, with PLGA/PEG scaffolds initially loaded with various masses of diclofenac sodium. (A) Representative images of osteoblast monolayers at day 7, live cells in green and dead cells in red. (B) Image quantification performed to determine average number of live and dead cells per image. Statistical significance of total cell number vs. total cell number in 0 µg/scaffold in osteogenic medium *($p \leq 0.01$). (C) Percentage of live cells in each group. Statistical significance vs. 0 µg/scaffold in osteogenic medium (**$p \leq 0.001$, ***$p \leq 0.0001$). Statistical significance vs. 0 µg/scaffold in cytokine medium (### $p \leq 0.0001$). Experiment repeated in triplicate, each with n=3.

Figure 5: Effect of diclofenac sodium releasing PLGA/PEG scaffolds on nitrite production in the *in vitro* calvarial osteoblast inflammation model. Nitrite accumulation was measured on day 1, 2 and 7, after scaffold placed in transwell. (A) Nitrite concentration at each timepoint. (B) Cumulative nitrite concentration over 7 days. Statistical significance vs. 0 µg/scaffold in osteogenic medium (*$p \leq 0.01$).

Statistical significance vs. 0 µg/scaffold in cytokine medium (#p≤0.01). Values represented as mean±SEM, experiment repeated in triplicate each with n=3.

Figure 6: Effect of diclofenac sodium releasing PLGA/PEG scaffolds on PGE_2 production in the *in vitro* calvarial osteoblast inflammation model. PGE_2 accumulation was measured on day 1, 2 and 7, after scaffold placed in transwell. (A) PGE_2 concentration at each timepoint. (B) Cumulative PGE_2 concentration over 7 days. Inset shows cumulative PGE_2 concentration excluding "no scaffold" groups. Statistical significance vs. 0 µg/scaffold in osteogenic medium (*p≤0.01). Statistical significance vs. 0 µg/scaffold in cytokine medium (#p≤0.01). Values represented as mean±SEM, experiment repeated in triplicate each with n=3.



Long term effect of diclofenac sodium on response of primary calvarial osteoblasts in proinflammatory cytokine medium. Osteoblasts were cultured for 14 days in osteogenic medium before experimentation. Cytokines (0.25 ng/mL IL-1β, 2.5 ng/mL TNF-α, 25 ng/mL IFN-γ) were applied throughout the 27 day period. (A) Cell viability, converted to percentage of control medium reading. Values represented as mean±SD, n=6. Experiment repeated in triplicate. (B) Cumulative nitrite concentration in culture medium at timepoints. Values represented as cumulative mean±cumulative SD, n=6. Experiment repeated in triplicate. (C) PGE2 concentration in medium at timepoints. Values represented as cumulative mean±cumulative SD. Experiment repeated in triplicate. For A, B, C: Statistical significance vs. control *(p≤0.01). Statistical significance vs. IL-1β+TNF-α+IFN-γ #(p≤0.01). (D) Representative immunocytochemistry images showing expression of osteopontin (OPN)/osteocalcin (OCN) and cadherin-11 (cad-11)/collagen-I (col-I) after treatment with IL-1β, TNF-α and IFN-γ and diclofenac sodium. Scale bar=90 μm.
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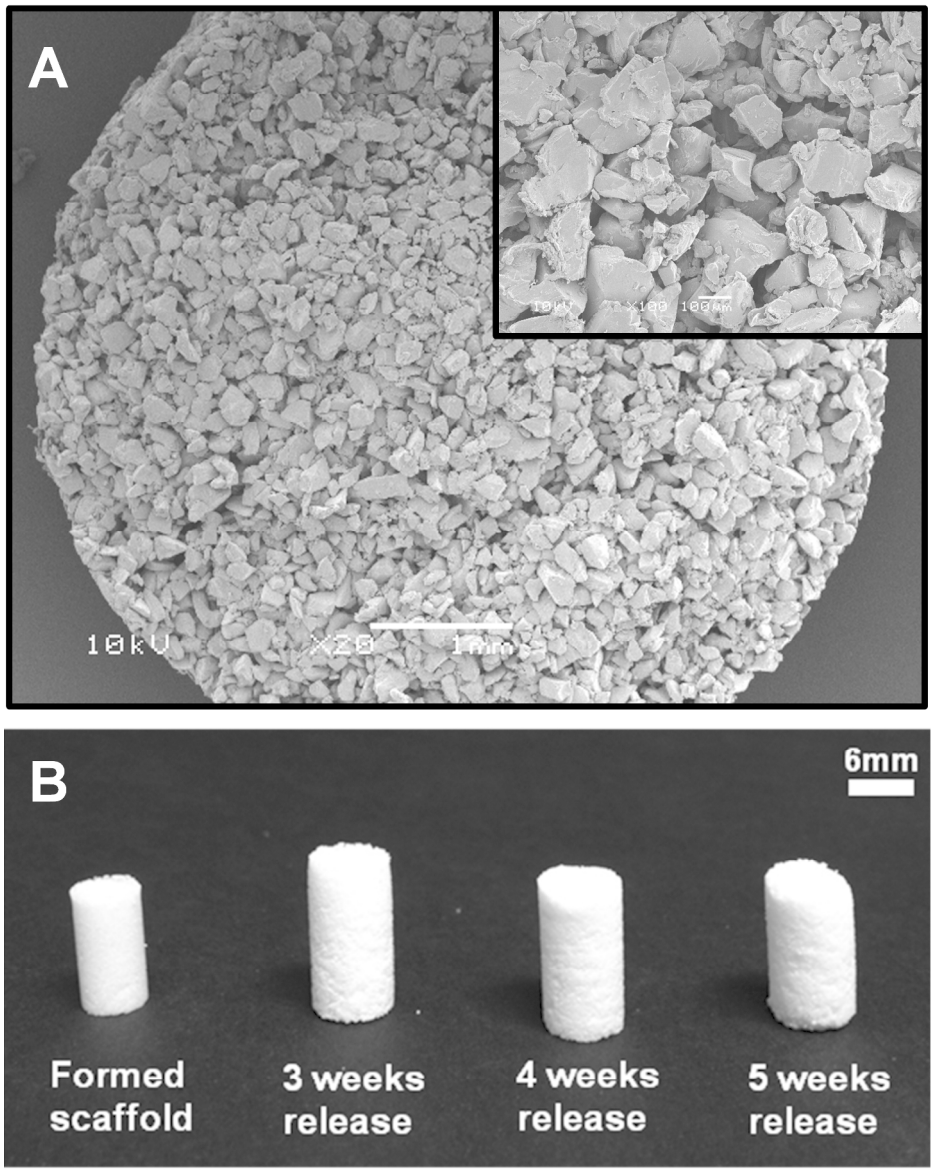

Figure 2: PLGA/PEG scaffolds. (A) Representative SEM images of scaffold microstructure. (B) Formed and sintered scaffolds showing swelling and degradation over 5 weeks.
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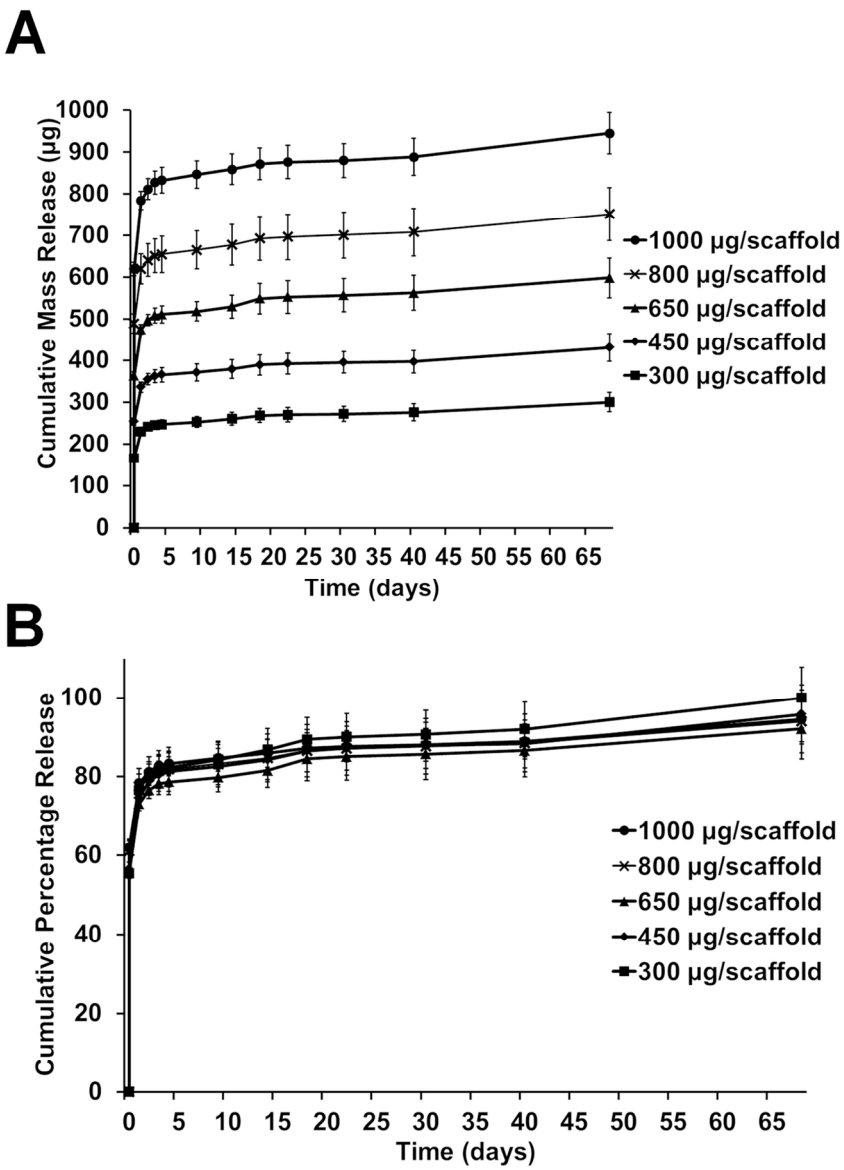

Figure 3: Diclofenac sodium release from PLGA/PEG scaffolds. Scaffolds were loaded with varying initial concentrations of diclofenac sodium. (A) Cumulative mass release of drug over time. (B) Cumulative percentage release of drug over time. Values represented by mean of triplicate scaffold release, each measured in triplicate, n=9. Error represented by cumulative SD.
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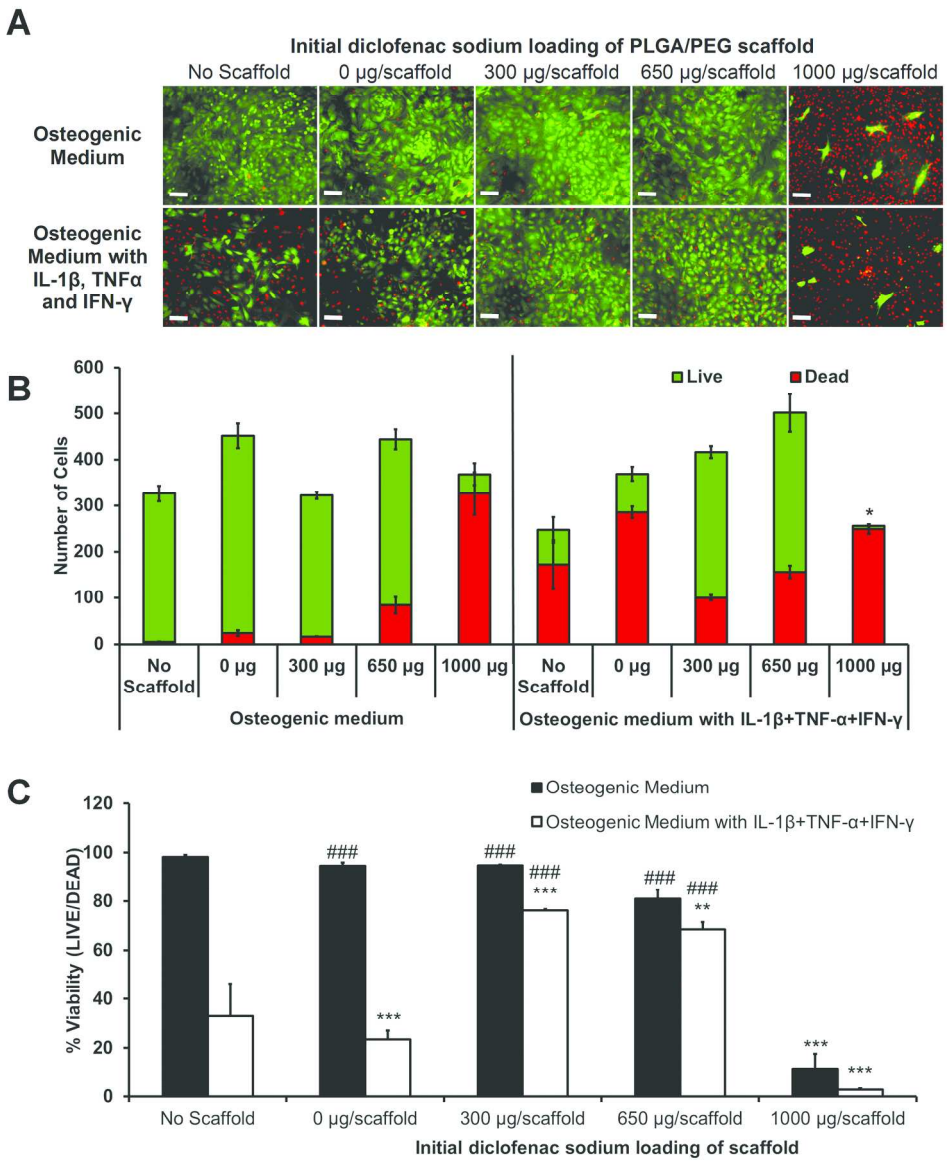

Effect of diclofenac sodium releasing PLGA/PEG scaffolds on cell viability in the in vitro calvarial osteoblast inflammation model. Live/Dead™ viability assay of osteoblast monolayers after 7 days culture with and without IL-1β, TNF-α and IFN-γ, with PLGA/PEG scaffolds initially loaded with various masses of diclofenac sodium. (A) Representative images of osteoblast monolayers at day 7, live cells in green and dead cells in red. (B) Image quantification performed to determine average number of live and dead cells per image. Statistical significance of total cell number vs. total cell number in 0 µg/scaffold in osteogenic medium *($p \leq 0.01$). (C) Percentage of live cells in each group. Statistical significance vs. 0 µg/scaffold in osteogenic medium (**$p \leq 0.001$, ***$p \leq 0.0001$). Statistical significance vs. 0 µg/scaffold in cytokine medium (### $p \leq 0.0001$). Experiment repeated in triplicate, each with n=3.
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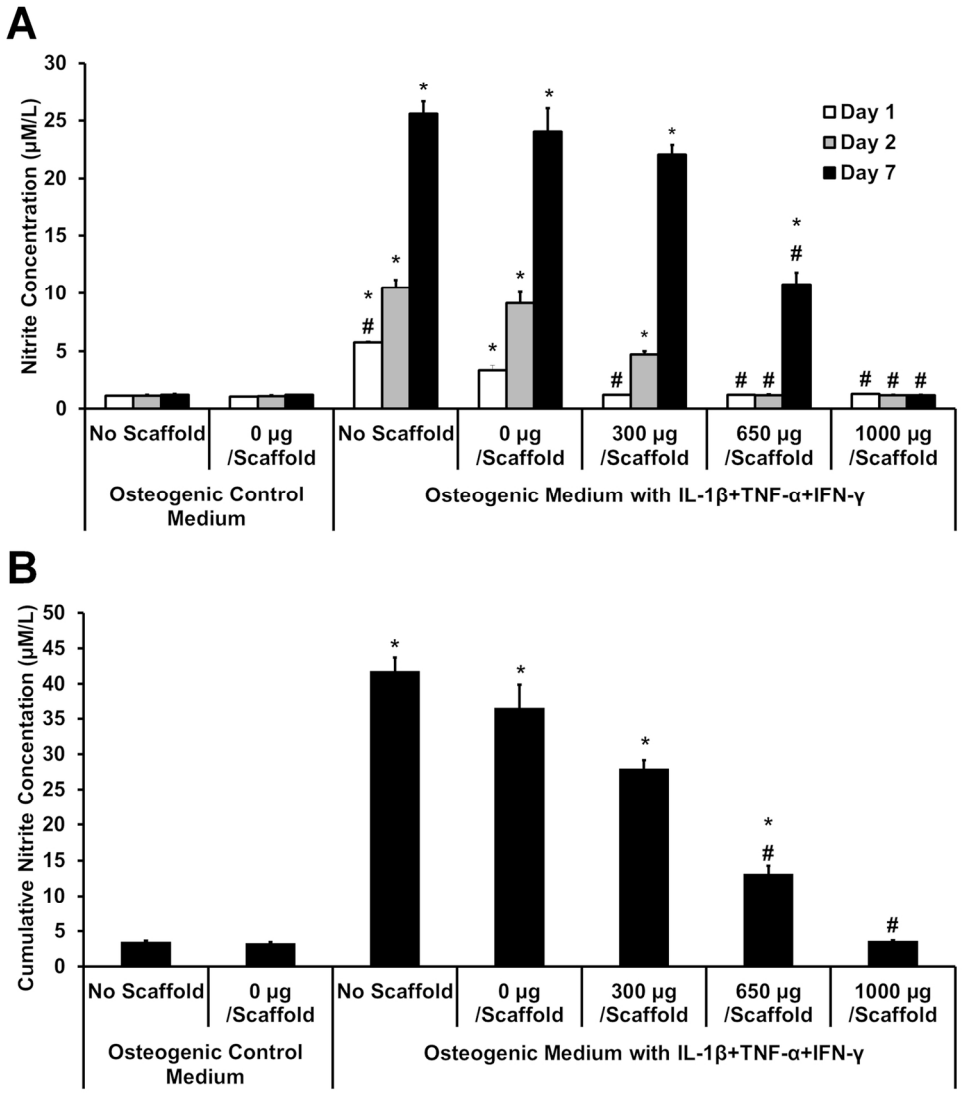

Effect of diclofenac sodium releasing PLGA/PEG scaffolds on nitrite production in the in vitro calvarial osteoblast inflammation model. Nitrite accumulation was measured on day 1, 2 and 7, after scaffold placed in transwell. (A) Nitrite concentration at each timepoint. (B) Cumulative nitrite concentration over 7 days. Statistical significance vs. 0 μg/scaffold in osteogenic medium (*p≤0.01). Statistical significance vs. 0 μg/scaffold in cytokine medium (#p≤0.01). Values represented as mean±SEM, experiment repeated in triplicate each with n=3.
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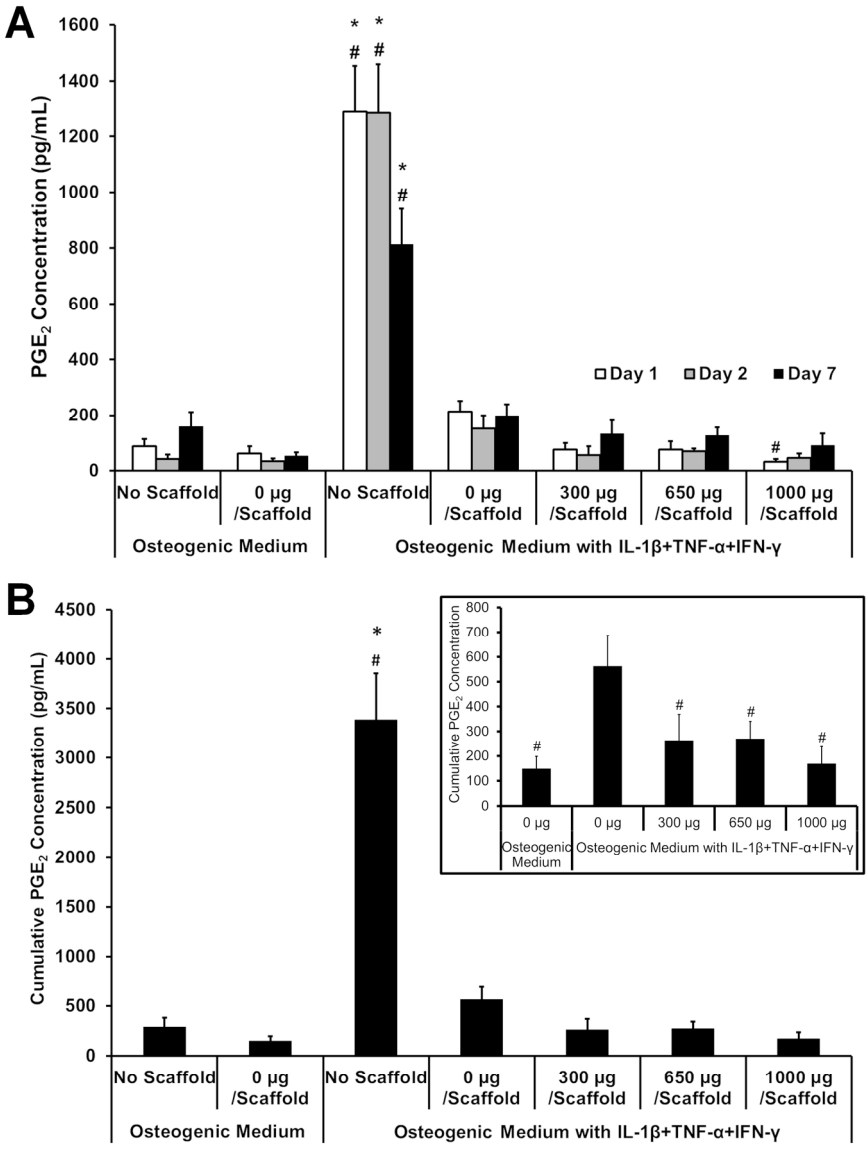

Effect of diclofenac sodium releasing PLGA/PEG scaffolds on PGE2 production in the in vitro calvarial osteoblast inflammation model. PGE2 accumulation was measured on day 1, 2 and 7, after scaffold placed in transwell. (A) PGE2 concentration at each timepoint. (B) Cumulative PGE2 concentration over 7 days. Inset shows cumulative PGE2 concentration excluding "no scaffold" groups. Statistical significance vs. 0 µg/scaffold in osteogenic medium (*p≤0.01). Statistical significance vs. 0 µg/scaffold in cytokine medium (#p≤0.01). Values represented as mean±SEM, experiment repeated in triplicate each with n=3.
162x215mm (300 x 300 DPI)

Response to Reviewers' Comments

Paper Title: Investigation of localised delivery of diclofenac sodium from PLGA/PEG scaffolds using an *in vitro* osteoblast inflammation model.

Authors: Laura E. Sidney, PhD; Thomas R. J. Heathman, MEng; Emily R. Britchford, MSc; Arif Abed, MEng; Cheryl V. Rahman, PhD; Lee D. K. Buttery, PhD.

The authors thank the reviewers for their helpful suggestions to improve the manuscript. We have endeavoured to respond to the points raised and make corrections to the manuscript where appropriate.

Author's responses are shown below the Reviewers' comments. Additions and corrections to the manuscript are highlighted yellow.

Reviewers' Comments to Author:
Reviewer: 1

General comment: *The paper presented by Laura Sidney et al, is a well written. The in vitro experiment design is reasonable. The pictures are clear. However, when compared to the papers Tissue Engineering Part A published, it is not sound enough. The contents are more suitable for material science or pharmaceutical science, since the authors did not perform any tissue engineering experiments even in vitro.*

Response: Thank you for your comments. The authors acknowledge that within this study, no "traditional" tissue engineering experiments were performed. However, we believe that the paper is suitable for publication in Tissue Engineering Part A as the PLGA/PEG scaffold is intended for a tissue engineering purpose (bone regeneration) with the additional function of anti-inflammatory release. Although the study does cover both drug delivery and biomaterials science, we consider these to be aspects of the wider tissue engineering field.

Comment 1: *No osteogenic experiment was performed when using scaffold as slow release system and compared to non-scaffold delivery. The reviewer suggests testing mineralization after release diclofenac with PLGA/PEG scaffold (such as Von Kossa or alizarin red staining).*

Response 1: We acknowledge the referees comment and suggestion. The cells were cultured in osteogenic medium for 14 days prior to being used in these experiments and we have shown previously that most effects of cytokines on osteogenic differentiation occur around this and earlier stages (Sidney et al., Stem cells and development, **23**, 65, 2014). We therefore chose to focus more on these stages rather than the end stage of differentiation and the bone nodule assay. Moreover, it is likely to be during the initial phases of exposure that effects will be manifest and subsequently carried through to later differentiation, again something we have shown previously.

Comment 2: *As PGE2 signal pathway is very important for bone regeneration and fracture healing in vitro and in vivo, suppress this pathway will impair the bone regeneration and bone healing. The initial inflammation after tissue injury is essential to initiate tissue repair process.*

Response 2: The authors agree that PGE_2, and the enzyme COX-2, play a crucial role in fracture healing. This study was designed as a model to show that the system is suitable for anti-inflammatory release but we are aware that a non-steroidal anti-inflammatory such as diclofenac may not be the most suitable drug, and a steroidal drug, specific COX-2 inhibitor or DMARD would be more suitable. Further *in vivo* testing would be required to investigate the long-term effects on fracture healing and unfortunately that is outside the scope of this *in vitro* investigation. The authors agree that the initial inflammation after tissue injury is crucial, and a balance would need to be sought between anti-inflammatory action to prevent capsule formation due to foreign body implantation, and the ability of the scaffold to assist bone regeneration.

Addition to manuscript: To clarify the point the reviewer made, we have added a short section to the discussion of the manuscript (page 17, paragraph 2) highlighting that PGE_2 also plays a role in fracture healing, alongside inflammation.

Comment 3: *The PLGA/PEG scaffold the authors investigated is widely studied.*

Response 3: One of the advantages of this study is that the PLGA/PEG formulation scaffold has already been established as suitable for bone regeneration, as discussed in the manuscript. However, this will be the first paper that describes the release of anti-inflammatory drugs from this system.

Comment 4: *The use of NSAIDs -diclofenac sodium is still controversial. Long term treatment could impair bone fracture healing, while short term treatment did not affect fracture healing, but do not enhance fracture healing either, therefore showing no beneficial effect (Krischack GD, Arch Orthop Trauma Surg. 2007 Jan;127(1):3-9. Epub 2006 Jul 25).*

Response 4: The authors agree with the reviewer that there is a lot of contradictory evidence showing the effects of NSAID treatment on fracture healing. We believe this study is an example of short term treatment, as although the scaffold does release drug for over 60 days, over 80% of release occurs within the first 4 days. The remaining drug is released at a state of about 0.2% per day. For the scaffold loaded with 650 μg drug this would be 1.3 μg per day. The authors do not know whether this concentration would have an effect on the local environment *in vivo*, but it is considerably below the dose administered per day in the publication stated. Assuming that this study can be considered short term administration, the fact that bone healing may not be adversely effected in the long-term, is a positive outcome. The addition of an anti-inflammatory drug was not intended to enhance fracture healing outside of the positive effect of the scaffold, but was envisioned to prevent any foreign body reaction or adverse inflammation upon implantation. As discussed above, diclofenac sodium or NSAIDs may not be the most suitable drugs for release *in vivo* but the system could be adapted to investigate drugs with alternative mechanisms of action.

Comment 5: *The reviewer thought the way the authors modified the data in Figure 6 by removing a comparison group was not appropriate. If looked at Figure 5, the use of scaffold also decreased the NO although the reviewer not sure there is a statistic difference. This might not be caused by the absorption of PGE2, rather than absorption of IL-1β, TNFα and IFN-γ since they are proteins.*

Response 6: The authors understand the reservations the reviewer has about the modifications of the data in Figure 6. However, the experiments could have as easily been performed without the "no scaffold" control and the important comparison in this study is the effect of the drug releasing scaffold in contrast to the no drug releasing scaffold. The "no scaffold" control was included to show the effect adding a scaffold had on the inflammatory model. It can be concluded that adding a scaffold changes the model by presenting a surface on which proteins can adsorb, including both the PGE_2 and proinflammatory cytokines. However, the model is still valid as a significant difference in PGE_2 is seen between scaffolds releasing diclofenac and non-releasing scaffolds. In figure 5, the scaffold group does have slightly lowered NO readings but this decrease is not significant. Due to this, the authors came to the conclusion that it is predominantly PGE_2 adsorption to the scaffold affecting results, but this may also be contributed to by cytokine adsorption to the scaffold. We have adjusted the presentation of the figure so that it shows PGE_2 release of all groups at each timepoint and as cumulative release, we have included an inset graph of the cumulative data without the "no scaffold" control to demonstrate that there is a reduction in the levels of PGE_2 when diclofenac is released.

Adjustment to manuscript: Figure 6 has been adjusted to include cumulative release with all data points. The results sections for figure 6 has been adjusted to explain this (page 15, paragraph 1) and a section added to the discussion to acknowledge that there were differences in the inflammation model when a scaffold was added (page 19, paragraph 3).

Comment 6: *Histogram in Figure 1 and 4 should be bigger for better viewing. Figure 1A should be indicated whether there are statistic differences between treatment and control groups regarding cell viability.*

Adjustment to manuscript: Both figure 1 and figure 4 have been adjusted to enlarge the histograms. Figure 1A has been adjusted to indicate the statistical differences between treated and control groups in terms of cell viability.

Comment 7: *Please do not use asterisk in control groups in histogram since the comparisons are always compared to control group and/or different treatment group. It is confusing to read.*

Adjustment to manuscript: Asterisks on control groups have been removed from figure 1, figure 4, figure 5 and figure 6.

Reviewer: 2

General comments: *This is a well organized and described study with a well supported rationale, detailed experimental plan with appropriate statistics throughout and the conclusions are supported by the results. A few minor comments follow.*

Comment 1: *In abstract - it would be nice to provide some indication of the magnitude of changes in most important cell parameters to make the abstract more informative/quantitative. This is important since the abstract is usually the first and often the only part of a manuscript read/available. Incorporating quantitative data will make the abstract more informative and better highlight the technical merits of the work for readers.*

Adjustment to manuscript: We thank the reviewer for their helpful comments. The abstract (pg 3) has now been adjusted to include some quantitative data about the cell parameters were affected by the introduction of the proinflammatory cytokines and the drug-releasing scaffolds, in terms of cell viability, NO production and PGE2 production.

Comment 2: *In the discussion section, it might be appropriate to comment on potential effects/role of diclofenac on macrophages/neutrophils since these cells are also critical to the inflammatory response - and in particular how diclofenac may influence macrophage phenotype (M1 vs M2).*

Response 2: Diclofenac sodium release would almost certainly have an effect on the presence and role of neutrophils and macrophages. However, the effect of this in bone regeneration is unclear as previous studies have indicated that an absence of neutrophils and macrophages have little effect on fracture healing, due to crossover of signalling between the inflammatory cells and the native bone cells. As the effect of diclofenac on inflammatory cells is of importance we have added a section to the discussion detailing the possible ramifications of diclofenac sodium release on macrophages and neutrophils.

Adjustment to manuscript: A section detailing the effect of diclofenac on neutrophil recruitment and macrophage activation has been added to the discussion on page 16, paragraph 2.