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Development and Biological Evaluation of Fluorophosphonate-Modified Hydroxyapatite for Orthopaedic Applications

Gráinne Neary[a*], Ashley W. Blom[a], Anna I. Shiel[b], Gabrielle Wheway[b] and Jason P. Mansell[b*]

[a] *Musculoskeletal Research Unit, University of Bristol, Level 1 Learning and Research Building BS10 5NB, UK.*

[b] *Department of Applied Sciences, University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol BS16 1QY, UK.*

[*]*Corresponding author email addresses:*

grainne.neary6@gmail.com, jason.mansell@uwe.ac.uk

Abstract

There is an incentive to functionalise hydroxyapatite (HA) for orthopaedic implant use with bioactive agents to encourage superior integration of the implants into host bone. One such agent is (3S) 1-fluoro-3-hydroxy-4-(oleoyloxy) butyl- 1-phosphonate (FHBP), a phosphatase-resistant lysophosphatidic acid (LPA) analogue. We investigated the effect of an FHBP-HA coating on the maturation of human (MG63) osteoblast-like cells. Optimal coating conditions were identified and cell maturation on modified and unmodified, control HA surfaces was assessed. Stress tests were performed to evaluate coating survivorship after exposure to mechanical and thermal insults that are routinely encountered in the clinical environment. MG63 maturation was found to be 3 times greater on FHBP-modified HA compared to controls ($p < 0.0001$). There was no significant loss of coating bioactivity after autoclaving ($P = 0.9813$) although functionality declined by 67% after mechanical cleaning and reuse ($p<0.0001$). The bioactivity of modified disks was significantly greater than that of controls following storage for up to 6 months ($p<0.001$). Herein we demonstrate that HA can be functionalised with FHBP in a facile, scalable manner and that this novel surface has the capacity to enhance osteoblast maturation. Improving the biological performance of HA in a bone regenerative setting could be realised through the simple conjugation of bioactive LPA species in the future.

Keywords: Hydroxyapatite; Lysophosphatidic acid; Biomaterial functionalisation; Bone graft; vitamin D.

1. Introduction

Hydroxyapatite (HA) is a widely used biomaterial in orthopaedic implant technology as a bone graft substitute [1, 2, 3, 4, 5] and as a coating for titanium implants [6, 7, 8, 9]. Traditional bone implant technologies have focused on the mechanical properties of implants to optimise their performance [10]. However, it is now known that the biological milieu has a significant impact on implant performance [11]. To this end, the use of composite orthopaedic implant devices supplemented with bioactive agents is a prominent theme in biomaterials research [12].

The term lysophosphatidic acid (LPA) is a moniker that refers to a diverse range of glycerophospholipids. The LPAs are lipid growth factors involved in a wide variety of cell functions [13, 14] including proliferation [15, 16, 17], differentiation [18, 19] migration [20, 21], apoptosis and survival [22, 23]. Importantly the LPAs have been shown to converge with active vitamin D3 metabolites to promote the maturation of osteoblast-like cells [24, 25]. These features of LPA, its small size and ability to co-operate with D3 make it an especially desirable molecule for implant functionalization.

LPAs sit within a class of compounds known as phosphonic acids (PA). Bisphosphonate drugs, which are used to reduce bone resorption in conditions such as malignancy and calcification disorders, are also members of the PA family. The ability of bisphosphonates to lock onto the mineral (HA) fraction of bone via their polar, phosphor head groups is widely recognised [26]. This ability to avidly bind HA was exploited by Torres and co-workers [27] who used an inert alkane PA, octadecylphosphonic acid (ODPA), for the subsequent addition of silver to create an antibacterial HA finish.

In this study, we explored the use of (3S)1-fluoro-3-hydroxy-4-(oleoyloxy)butyl-1-phosphonate (FHBP), a phosphatase-resistant LPA analogue, as a bioactive surface coating for HA. The effect of the FHBP coating on MG63 cell maturation and proliferation was assessed. Survivorship of the coating after autoclaving, storage, mechanical cleaning and reuse of the implant was determined. To our knowledge this is the first report of the use of a bioactive LPA analogue as a surface coating for HA for orthopaedic implant use.

2. Materials and Methods

2.1. Tissue Culture

Human osteoblast-like cells (MG63, ECACC, item code: 86051601) were cultured in conventional tissue culture flasks (750mL, Greiner, Frickenhausen, Germany) in a humidified atmosphere at 37°C and 5% CO_2. Cells were grown to confluence in Dulbecco's modified Eagle medium (DMEM)/F12 nutrient mix (Gibco, Paisley, Scotland) supplemented with sodium pyruvate (1mM final concentration), L-glutamine (4mM), streptomycin (100 ng/mL), penicillin (0.1 units/mL) and 10% v/v foetal calf serum (Gibco, Paisley, Scotland). The growth media was also supplemented with 1% v/v of a 100X stock of non-essential amino acids. For experimental purposes MG63 cells were seeded and maintained in the same growth medium but devoid of both serum and phenol red (SFCM).

2.2. Biomaterial optimisation

2.2.1. Determining the optimal FHBP concentration

HA disks (Solid Hydroxyapatite Discs, item code: HAD60, Hitemco Medical, USA) were autoclaved (121^0C for 30 minutes) prior to use. Two different sizes of disks were used over the course of the study; small disks with a surface area of 195.49mm^2 and large disks with a surface area of 304.02mm^2. Results are expressed per mm^2 to account for this difference in disk sizes. FHBP (LPA analogue, item code: L-9118-0.5mg, Tebu-bio Ltd., UK) was reconstituted to 500µM using 1:1 ethanol: cell culture-grade water and stored at - 20^0C. To ascertain the optimal FHBP steeping concentration to functionalise the HA specimens, sample disks were placed into 24-well tissue culture plates and exposed to varying concentrations of FHBP (0.25µM - 5µM) for a period of 24 hours. Cell culture-grade water was used as the diluent. Unmodified, control HA disks were immersed in cell culture-grade water.

Disks were removed from the wells after 24 hours and they were rinsed three times in cell culture-grade water before being left to air dry within the tissue culture hood. Once dry they were then transferred to a clean 24-well plate and seeded with MG63 cells. The optimal FHBP steeping concentration identified was used for all subsequent experiments. This test was performed in duplicate with n=4 per time point for each test.

2.2.2. Determining the optimal steeping time

Autoclaved HA disks were transferred to a fresh 24-well plate. 1ml of 2µM FHBP solution was added per well and disks were steeped for a specified time period (15 minutes, 1 hour, 2 hours, 4 hours or 24 hours) in the

solution. Control disks were steeped in the vehicle for the specified time period. Disks were removed from the wells after steeping and they were rinsed 3 times in cell culture-grade water before being left to air dry. Once dry they were transferred to a clean 24-well plate and seeded with MG63 cells. The optimal steeping time identified was used for all subsequent experiments. This experiment was performed in triplicate with n=4 per time point for each test.

2.3. Comparing cell maturation on FHBP-modified and control disks

HA disks were steeped in a 2µM solution of FHBP for 3 hours. Control disks were steeped in vehicle only. Modified and control disks were then rinsed 3 times in cell culture-grade water and left to air dry. Once dry the disks were transferred to a fresh 24-well plate and seeded with MG63 cells. This test was performed a total of 12 times with n=2-4 per group for each test.

2.4. Coating survivorship

2.4.1. Autoclave survivorship

HA disks were steeped in a 2µM solution of FHBP for 3 hours. One half of the batch of modified disks (n=3) was autoclaved at standard clinical settings (121^0C for 30 minutes) while the remaining half was not (n=3). Unmodified autoclaved disks served as a negative control. The disks were transferred to a clean 24-well plate and seeded with MG63 cells. This test was performed in triplicate with n=3-4 per group for each test.

2.4.2. Storage survivorship

HA disks were steeped in a 2µM solution of FHBP for 3 hours. Control disks were steeped in the vehicle only. Disks were either seeded with MG63 cells within 24 hours of modification or stored in air for a variable time period (6 week or 6 months) before being seeded with cells. Tests were performed in duplicate with n=6 per group for each time point for each test.

2.4.3. Mechanical cleaning and reuse

HA disks were steeped in 2µM FHBP solution for 3 hours. Control disks were steeped in the vehicle. The disks were seeded with MG63 cells and incubated under conventional issue culture conditions for 72 hours. After this initial use, the disks were mechanically cleaned with an electrical toothbrush (Proclinical™ C250, Colgate) whilst being held under running tap water for 10 seconds. They were then re-autoclaved (121^0C for 30 minutes) prior to a second use. This test was performed 4 times with n=3 per group for each test.

2.5. Biomaterial tissue culture model

For each of the above experiments disks were seeded with 1ml of a 150,000 cells/ml suspension of MG63 cells in SFCM spiked with calcitriol (D3) to a final concentration of 100nM [100nM, Calcitriol (10µg) (item code: D1530-10UG, Sigma)]. Plates were incubated under conventional tissue culture conditions for 72 hours before the alkaline phosphatase (ALP) activity of adherent cells was assessed.

2.6. Alkaline Phosphatase assay to compare cell maturation on FHBP-modified and control HA disks

After a 72 hour incubation period the medium in each well of a 24-well plate was removed. To ensure that the ALP activity was only associated with cells attached to HA and not to the surrounding plastic, the sample HA disks were transferred to a clean 24-well plate before being processed. An ALP substrate buffer was prepared according to Delory and King [28]**.** Briefly, 0.1M sodium carbonate and 0.1M sodium bicarbonate solutions were blended 7:3 to achieve a pH of 10.3. The resultant solution was supplemented with $MgCl_2$ to a final concentration of 1mM from a 1M stock solution. A lysis solution was prepared from this buffer by performing a 10-fold dilution using cell culture-grade water followed by spiking with Triton X-100 to a final concentration of 0.1% v/v. The monolayers on the surface of the HA disks were lysed with 0.1ml of this solution. After 2 minutes, each disk was treated with 0.2ml of 15mM p-nitrophenylphosphate (p-NPP, di-Tris salt, Sigma, UK) prepared in the ALP buffer. Lysates were left under conventional cell culturing conditions for 1 hour. After the incubation period, 0.1ml aliquots were transferred to a 96-well micro titre plates and the absorbance read at 405 nm. An ascending series of p- nitrophenol (p-NP) (50-500µM) prepared in the substrate buffer enabled quantification of product formation.

2.7. MTS/PMS assay to compare cell proliferation on FHBP-modified and control HA disks

HA disks were steeped in a 2µM solution of FHBP for 3 hours. Control disks were steeped in vehicle only. Disks were seeded with 1ml of a 150,000 cells/ml suspension of MG63 cells in SFCM spiked with D3 to a final concentration of 100nM. Plates were incubated under conventional tissue culture conditions for 72 hours, after which the medium from each well was removed and the HA disks were transferred to a fresh 24-well plate containing 0.5ml of SFCM per well. Cell number was determined using a combination of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, Promega, UK) and the electron-coupling reagent phenazine methosulphate (PMS). Each compound was prepared separately in pre-warmed (37 °C) SFCM, allowed to dissolve, and then combined so that 1 ml of a 1 mg/ml solution of PMS was combined to 19 ml of a 2 mg/ml solution of MTS. A stock suspension of MG63

cells (1 X 10^6 cells/ml) was serially diluted in SFCM to give a series of known cell concentrations down to 25 X 10^3 cells/ml to generate a standard curve. Each 0.5ml sample in the 24-well plate (HA disks) or in microcentrifuge tubes (standard curve samples) was spiked with 0.1 ml of the MTS/PMS reagent mixture and left for 45 min within a tissue culture cabinet. Once incubated, the medium surrounding the disks in the 24-well plate was harvested and 0.1ml of each sample was dispensed onto a 96-well microtitre plate and the absorbances read at 492 nm using a multiplate reader. Plotting the absorbances against known cell number, as assessed initially using haemocytometry, enabled extrapolation of cell numbers for the experiments described herein.

2.8. ALP gene expression analysis in MG63 cells

2.8.1. RNA purification

MG63 cells were grown to 80% confluency in T75 flasks and subsequently serum starved for 24 hours. 24 hours later, cells were treated with either media alone (control), D3 (100nM), FHBP (250nM) or vitamin FHBP/D3 in combination. After 24 hours of treatment, total RNA was extracted from cells using TRI reagent (Sigma Aldrich). RNA samples were treated with a TURBO DNA-free™ Kit (Ambion Inc.) using conditions recommended by the manufacturers, and then cleaned with an RNA Clean & Concentrator™-5 spin column (Zymo Research Corp). RNA was tested for quality and yield using a NanoDrop 1000 spectrophotometer and an Agilent 2100 Bioanalyzer. Three independent biological replicates were prepared (n = 12).

2.8.2. Library preparation

Twelve total RNA samples were supplied and prepared into sequencing libraries of ~500ng by Bristol Genomics Facility using the Illumina TruSeq Stranded mRNA kit. Briefly, RNA was polyA-selected, chemically fragmented to approximately 200 nt in size (4 minute fragmentation time), and cDNA synthesized using random hexamer primers. Each individual library received a unique Illumina barcode and pooled.

2.8.3. RNA sequencing

RNA-seq was performed on an Illumina NextSeq500 instrument with 12 libraries multiplexed and run across 4 lanes per flow-cell using 75 bp single end reads in high output mode. This resulted in more than 400 Million reads per flow cell, with an average of 38 million reads per sample.

2.8.4. RNA sequence analysis

Raw reads from 4 lanes per sample (4 FASTQ files) were aligned to the human (*Homo sapiens*) full genome (GRCh38/hg38) using STAR, a splice-aware aligner [29], with GRCh38.92.gtf gene model for splice junctions.

Again, using GRCh38.92.gtf, raw gene counts were estimated on merged BAM files using HTSeq, using the union method and –stranded=reverse options [30]. Differential gene expression was analysed using DESeq2 [31] and normalised read counts were obtained.

2.9. Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 7.00 software. Unless otherwise stated all data was subjected to a one-way analysis of variance (ANOVA) to test for statistical significance. A post hoc Tukey's multiple comparisons test was performed between all groups where a P-value of <0.05 was detected. An unpaired t-test (2-tailed) was used to compare means of modified and unmodified disks, and of freshly used and stored modified disks. P-values of <0.05 were considered significant.

3. Results

3.1. Biomaterial Optimisation

3.1.1. Optimal FHBP concentration

There was a significant increase in cellular ALP expression for all modified groups steeped in concentrations of ≥ 0.5µM FHBP solution compared to the unmodified control. There was no significant difference in ALP expression between the 0.5, 1, 2 and 5µM FHBP treatment groups. Therefore an "optimal" concentration of 2µM FHBP was empirically selected from this concentration range (**Fig1**).

3.1.2. Optimal FHBP steeping time

ALP expression was significantly greater for all FHBP-modified disks compared to control disks irrespective of steeping time. ALP expression was not significantly different following steeping for 2, 6 or 24-hour time periods. Therefore an "optimal" steeping time of 3 hours was empirically chosen from this range (**Fig2**).

The optimal conditions identified (2µM FHBP for 3 hours) were used for all subsequent experiments.

3.2. Comparing cell maturation on FHBP-modified and control disks

Cellular ALP activity was significantly greater for FHBP-modified disks (mean [p-NP] (µM) = 52.83 ± 34.71) compared to control disks (mean [p-NP] (µM) = 17.42 ± 5.934). This difference was significant ($p < 0.0001$) (**Fig3**).

3.3. Comparing cell proliferation on FHBP-modified and control disks

Cellular proliferation was significantly greater on FHBP-modified disks (mean cell number x1000=110.97 ± 38.8) compared to control disks (mean cell number= 76.3 ± 35.92). This difference was significant ($p= 0.0087$) **(Fig4).**

3.4. Autoclave survival

ALP expression did not significantly differ between the FHBP-modified autoclaved and non-autoclaved groups (mean [p-NP] (µM) = 58.83 ± 30.78 and 60.75 ± 27.98 respectively, $p= 0.9813$). The mean ALP activity of modified groups (autoclaved and non-autoclaved) was significantly greater than that of the control group ($p=0.0024$ and 0.0015 respectively) (**Fig5**).

3.5. Storage survival

The mean ALP activity of the modified stored groups was greater than that of the control groups after storage for 6 weeks and 6 months. This difference was significant at both time points ($p < 0.001$). This information is summarised in **Table 1**.

3.6. Mechanical Cleaning and Reuse

There was a 67% decline in cellular ALP activity for reused FHBP-modified disks compared to single use FHBP-modified disks (mean [p-NP] (μM) = 26.84 ± 11.74 and 81.56 ± 49.28 respectively). This difference was significant ($p = < 0.0001$). ALP activity did not significantly differ between the reused FHBP-modified group and the control (single use and reused) groups ($p = 0.9947$ and 0.8438 respectively) (**Fig6**).

3.7. ALP gene expression analysis

MG63 cells were treated in culture with either media alone (control), D3, FHBP, or a combination of FHBP and D3. Treatment with the FHBP/D3 combination resulted in a significant increase in normalised ALP gene expression compared to all other treatments ($p \leq 0.0001$), and, notably, a 7-fold increase in ALP expression compared to treatment with FHBP alone (**Fig7**).

4. Discussion

Autogenous bone grafts remain the gold standard in bone defect repair [32, 33]. However, there are issues associated with autogenous graft use, including donor site morbidity, limited tissue availability and prolonged operative time [34, 35]. Synthetic bone substitutes are being increasingly used in the clinical setting [32], providing an impetus to develop bone substitutes that satisfy the criteria of an "ideal bone graft".

Synthetic HA is a widely used bone substitute. It is a non-toxic compound with a similar chemical composition to human bone mineral [36, 37]. It is traditionally regarded as an osteoconductive material [35], providing a mechanical framework to support bone repair.

We have shown that coating HA with FHBP enhanced the maturation of MG63 cells in the presence of D3, thereby improving its osteoinductive properties. In agreement with this, MG63 cells that were co-treated with FHBP and D3 in tissue culture flasks showed elevated ALP gene expression. The MG63 cell line is an osteosarcoma-derived, transformed human cell line that exhibits features of the immature osteoblast, including trivial basal ALP expression. Maturation towards a mature osteoblast phenotype is associated with increased expression of ALP, an event that is synonymous with bone tissue formation [38, 39]. In the context of orthopaedic implants this is expected to improve osseointegration. Since our discovery that LPA and D3 co-operate to synergistically enhance osteoblast ALP expression we consistently find that the marked increase in this enzyme consequent to LPA/FHBP co-stimulation is not a result of raised cell number [40, 41] and therefore in keeping with a pro-differentiating function of this steroid hormone. We compared cell number between FHBP-modified and control HA disks. Although we observed an increase in cell number on FHBP-treated disks compared to control disks, this increase was modest (1.5x) compared to the increased ALP activity observed (3x). This suggests that the increased ALP activity observed was not simply a consequence of increased cell proliferation but also a consequence of accelerated cell maturation. This is further supported by our ALP gene expression data which provides compelling evidence for an increase in ALP gene expression for FHBP/D3 co-treated MG63 cells, an effect that has not been paralleled by an increase in cell number in our previous works [40, 41].

There are several reasons why we used an LPA analogue as a surface coating for HA. Firstly, LPA, in combination with D3, synergistically enhances human osteoblast maturation [24, 25]. LPA analogues are amenable to large scale production. Their natural affinity for HA simplifies manufacturing, and, because of their small size and lysophospholipid properties, they are robust molecules resistant to the rigours of implant handling

and sterilisation. Finally, antimicrobial properties of LPA family members have previously been reported [42, 43]. Although not investigated as part of this study, it has previously been shown that surface modification of titanium with an LPA analogue (16: 0 MPPA) deterred the attachment of methicillin-resistant *Staphylococcus aureus* to the implant surface [38]. Identifying strategies to prevent implant infection is a prominent theme in biomaterials research and has important public health implications in terms of reducing the reliance of modern medicine on antimicrobials.

In order to be considered for clinical applications, an implant coating must be safe, stable, resistant to sterilisation, amenable to reuse and resilient to mechanical forces. We have shown persistent bioactivity of the FHBP coating following ambient storage for 6 months. The coating survived autoclaving at standard clinical settings, and the coating process was performed as a single-step procedure under physiological conditions, thereby eliminating biocompatibility and cytotoxicity concerns. These features are expected to improve the translational prospects of this technology.

There was a significant loss of FHBP bioactivity following mechanical cleaning and reuse, which represents a limitation of its utility as an implant coating. Although implants are generally intended for single clinical use, there are a variety of circumstances in which they may need to be reused. Cleaning and sterilisation are key steps in reconditioning the implant to its initial state for reuse [44]. The reason for the loss of bioactivity is unclear and it contrasts with the findings of Mansell *et al.* [45] for their work on FHBP- and LPA-modified titanium. A possible explanation for this discrepancy relates to differences in the material properties and physical robustness of titanium and HA.

Failure to demonstrate successful adhesion and proliferation of MG63 cells on the modified HA disks represents a potential limitation of this work. However, HA is widely recognised as a biocompatible substance, hence its widespread use as a bone graft substitute and as a coating for metallic prostheses [46]. Indeed, in a previous work by us we have depicted osteoblasts firmly attached on the surface of HA disks together with evidence of proliferation in response to an LPA analogue [47].

Surface characterisation using techniques such as x-ray spectroscopy, scanning electron microscopy and/or atomic force microscopy, are commonly used to validate the presence of coatings on biomedical implants. However, we elected to focus on the identification of a biologically active surface, which we consider to be a more suitable test for a product that is ultimately intended for clinical applications.

This work represents a logical progression from the work of Torres et al. [27] who exploited the natural affinity of members of the phosphonic acids for HA by using ODPA as a linker molecule to attach silver ions. To our knowledge ours is the first report of the use of a phosphonic acid as a biomimetic coating for HA. Evaluating the antimicrobial potential of the coating and improving its resilience to mechanical stress and reuse are important next steps in the translational process.

5. Conclusions

To our knowledge this is the first report of successful functionalization of a HA construct intended for orthopaedic applications with FHBP to improve the biological properties of the implant. This was achieved in a facile and scalable manner. Accelerated maturation of osteoblasts is predicted to secure superior integration of the implant with host bone, thereby minimising implant micro motion. Further work is required to improve the survivorship of the coating to mechanical cleaning and reuse.

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Conflict of Interest

The authors declare that they have no conflict of interest.

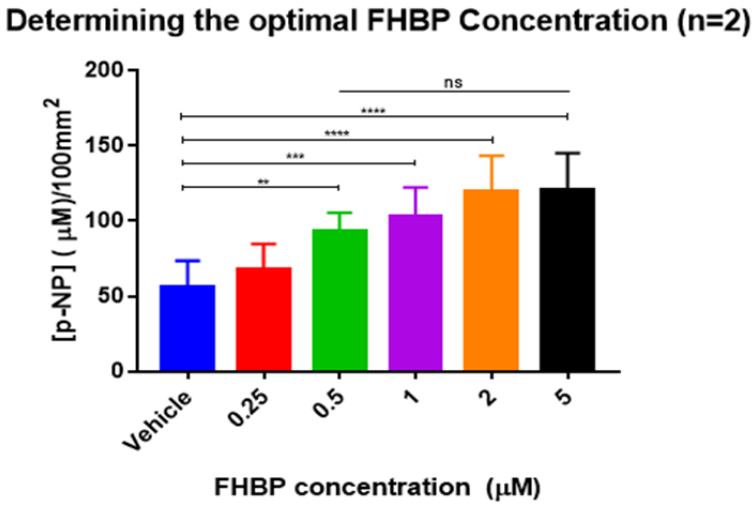

Fig1

The optimal concentration of FHBP solution to coat HA disks for enhanced MG63 maturation lies between 0.5-5µM. There was a significant increase in ALP expression for all treated groups compared to the control vehicle group at concentrations of ≥ 0.5µM FHBP. There was no significant difference in ALP expression between the 0.5, 1, 2 or 5 µM FHBP groups. The data depicted represent the mean and standard deviation of 4 replicates and are representative of 2 pooled experiments. Significant differences are depicted by asterisks (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, ns $P > 0.05$)

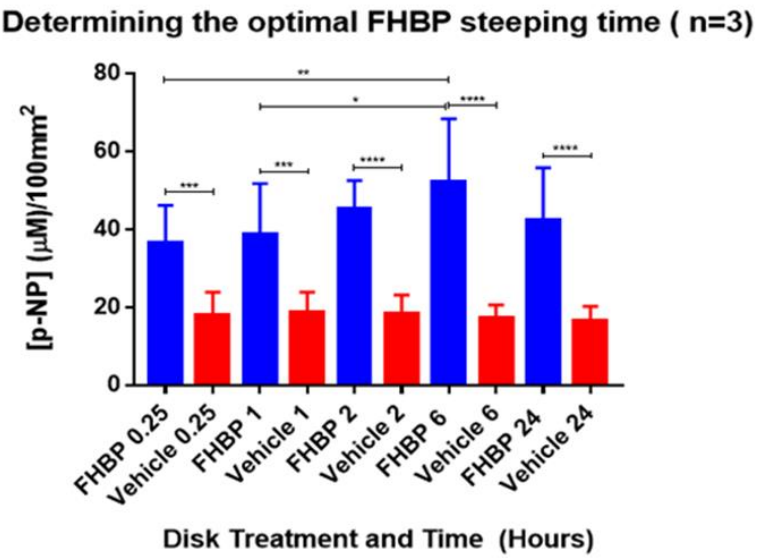

Fig2

The optimal steeping time in FHBP solution for enhanced maturation of MG63 cells on modified HA disks lies between 2-24 hours. ALP expression was significantly greater by MG63 cells adherent to FHBP-modified disks compared to unmodified disks at all time points investigated. There was no significant difference in ALP expression between the 2, 6 or 24-hour groups. The data depicted represent the mean and standard deviation of 3 (n=1) or 4 (n=2) replicates and are representative of 3 pooled experiments. Significant findings are depicted by asterisks (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns $P > 0.05$)

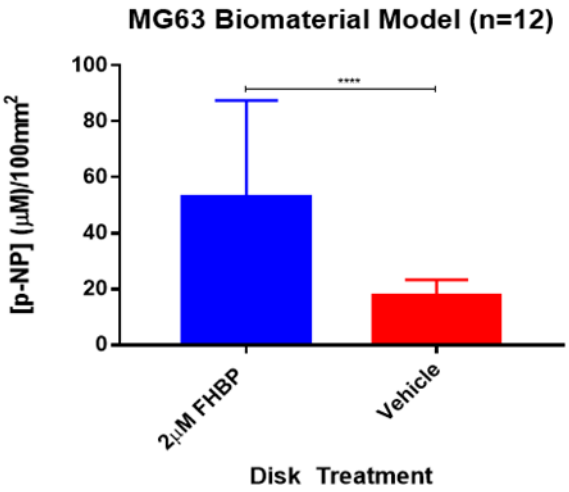

Fig3

Modification of HA disks with a surface coating of FHBP accelerates the maturation of adherent MG63 cells. This figure shows that the ALP activity of MG63 cells adherent to the surface of a HA disk that had been modified by a surface coating of 2µM FHBP was greater than that of MG63 cells adherent to the surface of an unmodified HA disk. The data depicted represent the mean and standard deviation of a minimum of 2 replicates and are representative of 12 pooled experiments Significant difference are depicted by asterisks (**** $P \leq 0.0001$)

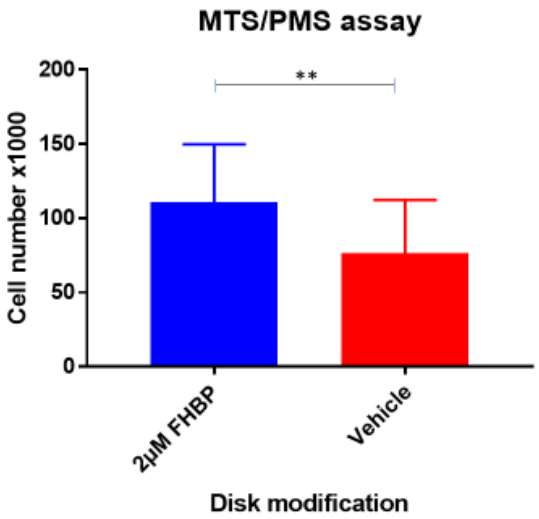

Fig4

Modification of HA disks with a surface coating of FHBP promotes cell proliferation. This figure shows that surface modification of HA disks results in an increased number of adherent MG63 cells. However the increase in cell number is modest compared to the increased cell maturation that is observed when MG63 cells are grown on FHBP-modified HA disks (see Fig3). The data depicted represent the mean and standard deviation and are representative of 3 independent experiments with 6 replicates per group. The significant difference is depicted by an asterisk (** $P \leq 0.01$).

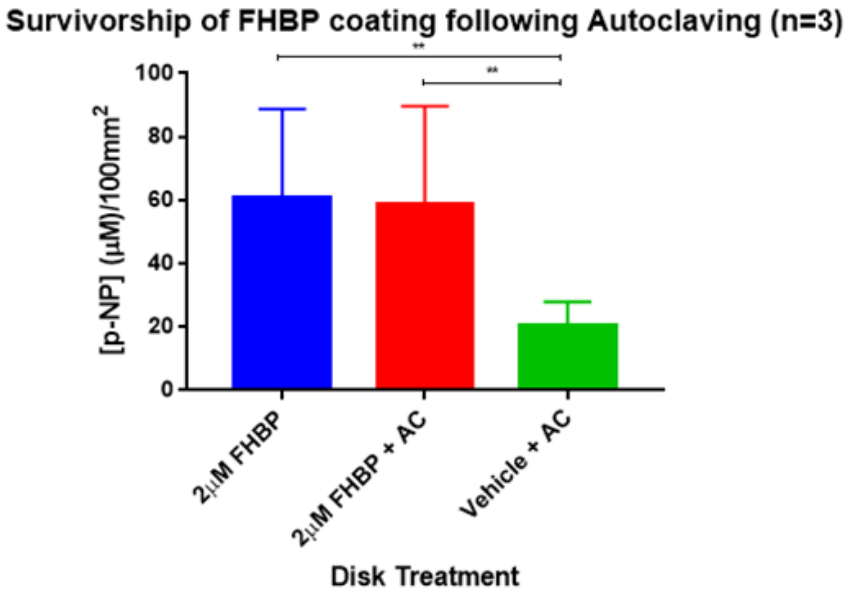

Fig5

The FHBP coating can withstand autoclaving at standard clinical settings. There was no significant difference in ALP activity between MG63 cells adherent to autoclaved (AC) or non-autoclaved FHBP-modified disks. The data depicted represent the mean and standard deviation of a minimum of 3 replicates and are representative of 3 pooled experiments. Statistical significance is depicted by asterisks (** $P \leq 0.01$)

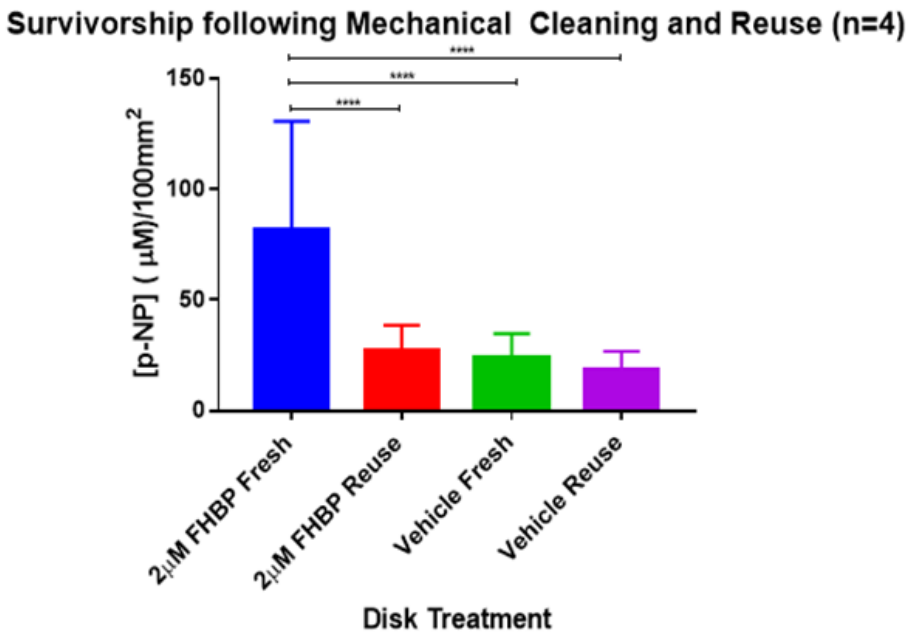

Fig6

The bioactivity of the FHBP coating is lost following mechanical cleaning, irrigation and reuse. This figure shows that there was a significant decline in cellular ALP activity once the FHBP-modified HA disk had been mechanically cleaned and reused compared to single use FHBP- modified disks. The data depicted represent the mean and standard deviation of 3 replicates and are representative of 4 pooled experiments. Significant results are depicted by asterisks (**** $P \leq 0.0001$)

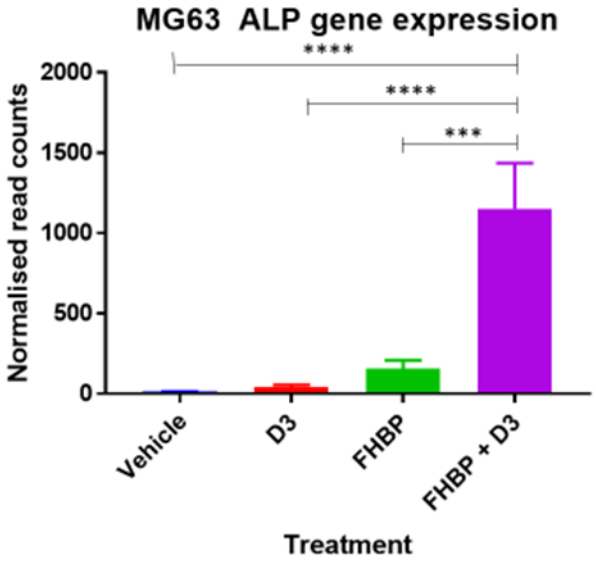

Fig7

Co-treatment of MG63 cells in tissue culture with FHBP and D3 results in a significant increase in ALP gene expression compared to treatment with D3, FHBP, or vehicle. Significant differences are depicted by asterisks (**** $P \leq 0.0001$, *** $P = 0.0001$).

Note: All figures shown have been created using GraphPad Prism 7.00 software.

Time	Vehicle [p-NP] (µM)	2µM FHBP [p-NP] (µM)	P-Value (Test versus Control)
Baseline	75.81 ± 22.14	121.52 ± 25.92	0.0017*
6 weeks	69 ± 12.97	165.67 ± 50.47	<0.0001*
6 months	16.92 ± 9.8	108.36 ± 55.13	<0.0001*

Table 1: Summary of the osteoblast ALP activity at control and FHBP-modified HA surfaces – a six month ambient storage study. At the specified times Human (MG63) osteoblasts were seeded on to control and FHBP-functionalised HA discs at a density of $15x10^4$ cells/ml/disc in the presence of 100nM D3. After a three day conventional culture the discs were processed for total ALP activity using p-nitrophenylphosphate as the substrate and quantification of p-nitrophenol (p-NP). An increase in p-NP for the FHBP-HA discs reflects a greater extent of osteoblast maturation at these surfaces compared to non-functionalised controls. This difference in mean ALP activity was significant at all time points investigated ($p<0.05$). Significant differences are depicted by asterisks.