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(54) Title: ORTHOPAEDIC MATERIAL

(57) Abstract: There is provided an orthopaedic material comprising a biocompatible material such as titanium and at least one organic LPA receptor agonist compound,the compound comprising at least one inter-carbon double bond, the compound being covalently bound to a surface of the biocompatible material via a carbon in the double bond. There is also provided a method of making such a material.

Orthopaedic Material

Field of the Invention

An improved orthopaedic implant material is provided, particularly a titanium material having a lysophosphatidic acid (LPA) receptor agonist compound such as 1-oleoyl LPA or FHBP (1-fluoro-3-hydroxy-4-butyl-1-phosphonate) covalently attached thereto. Such implant materials promote osteoblast maturation at the implant surface.

Background

Osteoblast formation and maturation at prosthetic surfaces is predicted to enhance biomaterial integration and ultimately implant longevity. Realising the fabrication of superior implant materials includes identifying molecules that are reported to promote human osteoblast development and differentiation at implant surfaces. Closely associated with identifying suitable bioactives, are identifying appropriate strategies for attachment of selected molecules to the implant material. Although a large number of studies have focussed on the use of non-covalent interactions of various agents to biomaterials, the longevity, efficiency and suitability of non-covalent attachment in high-performance environments such as total joint replacements is unlikely to succeed.

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate; LPA) is the trivial name assigned to a variety of simple glycerophospholipids composed of a fatty acyl chain, glycerol backbone and a phosphate head group. Different LPAs vary according to acyl chain length (for example, 15, 16, 17, 18, 19 or 20 carbon atoms) and degree of saturation (for example, 0, 1, 2, 3, 4 or 5 inter-carbon double bonds). Typically, 1-oleoyl LPA (18:1-LPA, having 18 carbon atoms and one inter-carbon double bond) is the species used in describing cell and tissue responses to this lipid and this also extends to skeletal cells.

At present there are seven cell surface receptors for LPA. Of these, LPA receptors 1-4 (known as LPA1, LPA2, etc.) are considered to be the most significant for skeletal cells. Human osteoblasts express LPA1 and LPA3 while marrow stromal cells express LPA1 and LPA4, with low variable expression of LPA2 and LPA3 (Mansell *et al.* (2011) Prostaglandins and other Lipid Mediators vol. 95 p 45-52).

Recently, the present inventors reported that 18:1-LPA and the LPA1/3 receptor agonist, OMPT, synergistically co-operated with calcitriol (D3) to promote human MG63 osteoblast maturation at both titanium (Ti) and hydroxyapatite (HA) surfaces (Mansell *et al.* (2010) Biomaterials vol. 31 p 199-206). Lysophosphatidic acid and related lipids lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P) and the S1P receptor agonist prodrug, FTY720, are assuming a growing significance in mineralising tissue biology; LPC is a major circulating lipid precursor to LPA that has recently been reported to induce the transdifferentiation of human aortic smooth muscle cells to the osteoblast phenotype (Vickers *et al.* (2010) Atherosclerosis vol. 211 p 122-129). Both LPA and S1P have been identified as stimulating both human osteoblasts (Dziak *et al.* (2003) Prostaglandins, Leukotrines and Essential Fatty Acids vol. 68 p 239-249) and their bone marrow progenitors. These events likely interact to aid cell migration and survival, key events to securing tissue repair/regeneration (P'ebay *et al.* (2007) Prostaglandins and other Lipid Mediators vol. 84 p 83-97). Furthermore, recent studies have now revealed that FTY720, licensed for treating multiple sclerosis (Choi *et al.* (2011) Proc. Natl. Acad. Sci. U.S.A. vol. 108 p 751-756), is capable of securing superior neovascularisation, mechanical stability and osseointegration of large tibial bone allografts in rats (Aronin *et al.* (2010) Biomaterials vol. 31 p 6417-6424). The same compound has also been reported to aid cranial bone defect repair via the recruitment of local bone progenitors (Aronin *et al.* (2010) Tissue Eng. Part A vol. 16 p 1801-1809). It would appear, therefore, that low molecular mass lipids may be attractive candidates in a bone regenerative setting.

Summary of invention

According to a first aspect of the invention there is provided an orthopaedic material comprising a biocompatible material and at least one organic LPA receptor agonist compound, the LPA receptor agonist compound comprising at least one inter-carbon double bond and being covalently bound to a surface of the biocompatible material via a carbon in the double bond. The term "organic LPA receptor agonist compound" indicates that LPA receptor agonist compound comprises at least carbon and hydrogen atoms.

The LPA receptor agonist compound is, therefore, a compound which comprises at least one inter-carbon double bond prior to covalent binding to the surface of the

biocompatible material. The LPA receptor agonist compound may be covalently bound to the surface using, for example, an addition reaction as described elsewhere herein.

An orthopaedic material is one which is suitable for use to make implant structures such as replacement joints for implantation into a human or animal body during partial or complete arthroplasty. A biocompatible material is any known in the art for the purposes of arthroplasty such as titanium, titanium alloy, stainless steel, tantalum, tantalum alloy, polyethylene, hydroxyapatite, a natural polymer material or a saturated aliphatic polymer material, or derivatives of any of these. Therefore, rigid materials are generally intended and non-rigid materials such as agarose beads are not considered to be biocompatible materials. A material, particularly a metal, having an oxide layer formed on its surface may be especially suitable for the purposes of the present invention, for use to make the orthopaedic material according to the invention.

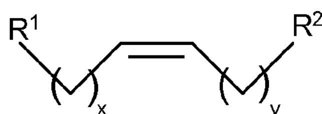
Given the discovery that 1-oleoyl LPA and calcitriol (D3) co-operate to stimulate human osteoblast differentiation, the development of an LPA-functionalised Ti surface was the next step in the inventors' ongoing programme of research aimed at enhancing the experience of arthroplasty. In this regard, the inventors hypothesised that an LPA-functionalised Ti surface could co-operate with local concentrations of D3 to heighten the maturation potential of attached osteoblasts and, with it, a greater potential to enhance initial implant integration into host tissue.

It was thought by the inventors that suitable candidate molecules ideally should be covalently attached to the material surface so that they withstand, for example, the shear stresses that arise during implantation whilst, importantly, retaining their biological activity. Another key consideration is size; smaller molecules will cover greater areas than much larger polypeptide/protein growth factors, e.g., bone morphogenic proteins (BMPs) and they are less likely to pose sterical hindrances to target cell receptors.

In developing an LPA-functionalised Ti surface it is important to ensure the correct alignment of LPA molecules in relation to the metal surface; phospholipids and phosphopeptides have a natural affinity for Ti (Larsen *et al.* (2005) Mol. Cell. Proteom. vol. 4 p 873-886). This non-covalent interaction has been exploited in the

fabrication, for example, of solid phase extraction systems to remove phospholipids from complex biological samples, including foodstuffs, e.g., chocolate milkshake (Calvano *et al.* (2009) Anal. Bioanal. Chem. vol. 394 p 1453-1461). However, the phospholipid-Ti interaction is via the polar head group and such a configuration is unsuitable in the generation of LPA-signalling supports, as it is the phosphate-group that binds to cell surface receptors to initiate cell signalling (Tigyi & Parrill (2003) Prog. Lipid Res. vol. 42 p 498-526). To ensure that the LPA is appropriately bound, the inventors targeted the alkene moiety in the tail section for covalent attachment to the metal surface. The present application describes how the covalent attachment of phospholipids such as 1-oleoyl LPA or a recently developed LPA3 receptor agonist, 1-fluoro-3-hydroxy-4-butyl-1-phosphonate (FHBP), produces a Ti surface that supports superior human osteoblast maturation to calcitriol. Of the materials produced in the inventors' laboratory, FHBP-functionalised Ti was the most superior at supporting calcitriol-induced human osteoblast maturation.

In the orthopaedic material according to the invention, therefore, the LPA receptor agonist compound may comprise the motif of Formula I:

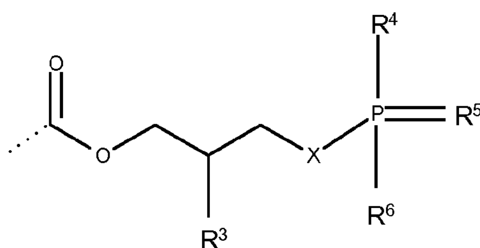


Formula I

where, for example, R^1=Formula II below, R^2=CH_3 and x=y or x≠y.

In certain embodiments, x=y=7 and R^2=CH_3.

Therefore, the LPA receptor agonist compound may comprise the motif of Formula II:



Formula II

where R^3 is OH or OR , R^4 is OH, Na^+O^- or OR, R^5 is O or S, R^6 is OH, Na^+O^- or OR and X is O or CHY (where Y is halide, for example, F); in all cases, R within OR is alkyl, such as CH_3.

In one embodiment (for example, 1-oleoyl-2-methyl-*sn*-glycero-3-phosphothionate;
5 OMPT), R^3 is OCH_3, R^4 and R^6 are OH, R^5 is S and X is O.

In another embodiment, (for example, FHBP), X is CHF, R^3 is OH, R^5 is O and R^4 and R^6 are Na^+O^-.

In another embodiment (for example, lysophosphatidylmethanol; LPM), R^3 and R^6 are OH, R^4 is OCH_3, R^5 and X are O.

10 In another embodiment (for example, 1-oleoyl LPA), R^3, R^4 and R^6 are OH, R^5 and X are O.

A LPA receptor agonist compound is any compound comprising at least one inter-carbon double bond which is capable of acting as a LPA receptor agonist, i.e., initiating at least one signalling cascade resulting from LPA receptor activation.

15 The skilled person may determine whether or not a compound is capable of acting as a LPA receptor agonist using a number of methods known in the art, for example, such as is disclosed in Gidley *et al.* (Prost. & other Lipid Mediators (2006) vol. 80 p 46-61). By way of example, the skilled person may prepare a culture of MG63 cells as outlined in section 2.2 of Gidley *et al.*, treat cells with a combination of D3 and the
20 compound which is being investigated for LPA receptor agonist activity and detect alkaline phosphatase (ALP) expression as described in section 2.7 of Gidley *et al.* An increase in ALP expression under these conditions, the increase being inhibited by Ki16425 (a known LPA receptor antagonist), is indicative of the compound being a LPA receptor agonist. Detection of Erk phosphorylation in the cells, as described in
25 section 2.9 of Gidley *et al.*, provides a further supporting indication of LPA receptor agonist activity of the compound.

Agonists of LPA1 and LPA3 are preferred, more preferably agonists of LPA1. Suitable compounds may be pan-agonists, i.e., agonists of more than one of, or all of, LPA1-4.

The LPA receptor agonist compound may be a phospholipid (i.e., a lipid typically comprising a glyceride and a phosphate group), for example comprising about 10-25, for example, 15-20 carbon atoms, such as about 15, 16, 17, 18, 19 or about 20 carbon atoms. The phospholipid may comprise at least one inter-carbon double bond, for
5 example 1-18 inter-carbon double bonds, such as about 1, 2, 3, 4 or 5 inter-carbon double bonds. Compounds with a single inter-carbon double bond are preferred.

The motifs of Formulae I and II above are found in all of the LPA receptor agonist compounds 1-oleoyl LPA (also called 18:1-LPA), 1-fluoro-3-hydroxy-4-butyl-1-phosphonate (FHBP), 1-oleoyl-2-methyl-*sn*-glycero-3-phosphothionate (OMPT) and
10 lysophosphatidylmethanol (LPM) and particular embodiments of the material according to the invention may comprise any one or more of these, covalently bound (via a carbon from the inter-carbon double bond) to a surface of the biocompatible material. Other compounds comprising the motif of Formula I (including that of Formula II) are encompassed within the term "LPA receptor agonist compound" as
15 used herein.

In particular embodiments, the LPA receptor agonist compound may be 1-oleoyl LPA or FHBP.

As mentioned above, the biocompatible material may comprise titanium, a titanium alloy, stainless steel, tantalum, a tantalum alloy, polyethylene, hydroxyapatite, a
20 natural polymer material (e.g., a collagen-, fibrin-, agarose- or chitosan-based material) or a saturated aliphatic polymer material (e.g., poly(lactic acid), poly(glycolic acid), poly(lactic-coglycolide)), or derivatives of any of these. Materials comprising an oxide layer on the surface may be especially suitable. In a particular embodiment, the biocompatible material comprises titanium.

25 The material may comprise 10-60% surface area coverage of the LPA receptor agonist compound, that is, 10-60% or 20-50% or 30-50% of the theoretical maximum coverage calculated as set out in Appendix A herein. For example, the material may comprise at least about 10%, 15% 20%, 25%, 30%, 35%, 40%, 45%, 50% or at least about 55% the theoretical maximum coverage, for example about 41%, 42%, 43%,
30 44%, 45%, 46% or about 47% the theoretical maximum coverage.

The material may be characterised in that it promotes calcitriol-induced osteoblast maturation (for example, human osteoblast maturation) as compared to the maturation obtained using the biocompatible material which does not have a LPA receptor agonist compound covalently bound to a surface thereof, i.e., osteoblast maturation (occurrence or rate) is increased when contacting the osteoblasts with the material according to the invention. Alternatively or additionally, it may promote formation of osteoblasts from marrow-derived stromal cells (e.g., hBMSCs) and mineralisation, as compared to the formation achieved using the biocompatible material which does not have a LPA receptor agonist compound covalently bound to a surface thereof, i.e., osteoblast formation from marrow-derived stromal cells (occurrence or rate) is increased when contacting the cells with the material according to the invention. Surprisingly, these properties of the material are still present even after one, two, three or more rounds of washing and/or scrubbing of the material, which might be expected to remove a proportion of the bound LPA receptor agonist compound.

The material of the invention may be formed as an orthopaedic implant device or portion thereof, i.e., an implant device or portion thereof may be formed from (or at least partially using) the orthopaedic material according to the invention. Composite devices comprising the material according to the invention in combination with other materials are envisaged. An orthopaedic implant device is any device intended for insertion, on a temporary or permanent basis, into a body to replace or repair or abut a component of the body which is formed from bone. By way of non-limiting example, the device may be a replacement joint (e.g., a knee, hip or knuckle) or portion thereof, a replacement long bone (e.g., tibia, fibula, radius, ulna, femur, humerus) or portion thereof, a replacement vertebra or portion thereof, or a pin, bolt or screw intended for engagement with or insertion into or through a bone. The body may be an animal body, for example a mammal such as a human, cat, dog or horse.

According to a second aspect of the invention there is provided a method of obtaining a material according to the first aspect of the invention, comprising the steps of:

a) immersing or substantially coating a biocompatible material item (e.g., an orthopaedic implant device or a disc for use in cell culture, for example, a titanium item) in Piranha solution at room temperature, or in 10M NaOH at elevated temperature, for example, about 60°C;

b) washing the item with deionised water and drying the item;

c) immersing or substantially coating the item in anhydrous organic solvent;

d) adding a linker molecule;

e) rinsing the item in anhydrous organic solvent and drying the item;

5 f) adding a stock solution of an organic LPA receptor agonist compound in anhydrous organic solvent;

g) adding a radical initiator and exposing the item to a reaction initiator;

h) rinsing the item in anhydrous organic solvent;

i) drying the item and optionally storing before use;

10 wherein step (d) comprises either the step of adding a reactive intermediate such as (3-mercaptopropyl) trimethoxysilane (MPTMS) and proceeding to step (e), or the steps of adding a difunctional linker such as vinyl trimethoxysilane (VTMS), rinsing the item in anhydrous organic solvent, adding anhydrous organic solvent supplemented with 1,8 octanedithiol, adding a radical initiator and exposing the item 15 to a reaction initiator and proceeding to step (e).

The organic solvent may be toluene. Steps involving addition of a radical initiator and exposing the item to a reaction initiator may comprise adding azobisisobutyronitrile (AIBN) and heating at elevated temperature, for example, about 60°C. Steps involving addition of a radical initiator and exposing the item to a reaction initiator 20 may alternatively comprise adding a UV radical initiator such as benzoyl peroxide and irradiating with UV radiation.

In a particular embodiment, step (d) comprises the step of adding (3-mercaptopropyl) trimethoxysilane (MPTMS) and proceeding to step (e).

The immersion or coating in step (a) may continue for 0.5 to 5 hours, for example 1 to 25 4 hours, for example about 1.5, 2 or 2.5 hours. In step (d), the item may be immersed or coated in MPTMS or VTMS for 12-36 hours, for example 20-30 hours, for example about 23, 24 or 25 hours. In step (d) and/or step (g), the item is immersed or coated in AIBN and heated at about 60°C for a period of 5-15 hours for example, 7-12 hours, for example about 8, 9 or 10 hours, for example, overnight.

30 The Piranha solution may be formed by combining equal volumes of ice-cold concentrated sulphuric acid and about 30% (w/v) hydrogen peroxide, mixing for 5-15

minutes, for example, for about 10 minutes and allowing the mixture to come to room temperature prior to use.

The stock solution of an organic LPA receptor agonist compound may be a stock solution of a compound which comprises at least one inter-carbon double bond and which is capable of acting as a LPA receptor agonist, as defined above. For example, the stock solution may be a stock solution of at least one phospholipid such as one or more of 1-oleoyl LPA, 1-fluoro-3-hydroxy-4-butyl-1-phosphonate (FHBP), 1-oleoyl-2-methyl-*sn*-glycero-3-phosphothionate (OMPT) and lysophosphatidyl methanol (LPM). A LPA stock solution may be formed by preparing a solution of 1-oleoyl LPA at a concentration of about 10mM in about 1:1 ethanol:tissue culture grade water. Likewise, a FHBP stock solution may be formed by preparing a solution of FHBP at a concentration of about 10mM in about 1:1 ethanol:tissue culture grade water.

An orthopaedic device formed from the material of the first aspect of the invention and/or material made using the method of the second aspect of the invention may be for use in a method of implanting a surgical device into a human or animal body.

Likewise, a method of implanting a surgical device into a human or animal body comprising implanting an orthopaedic device formed from the material of the first aspect of the invention and/or material made using the method of the second aspect of the invention.

According to a further aspect of the invention, there is provided a method of promoting maturation of osteoblasts on a surface comprising forming the surface from an orthopaedic material according to the first aspect of the invention (or made using the method of the second aspect of the invention) and contacting the surface with a sample of osteoblast cells (for example, human osteoblast cells such as MG63 cells. The method may comprise contacting the surface and osteoblasts with calcitriol (D3). There is also provided a method of promoting formation of osteoblasts from marrow-derived stromal cells on a surface comprising forming the surface from an orthopaedic material according to the first aspect of the invention (or made using the method of the second aspect of the invention) and contacting the surface with a sample of marrow-derived stromal cells (e.g., hBMSCs). Each of these methods may be an *in*

vitro cell culture method or an *in vivo* method of promoting osteoblast maturation and/or formation around a device implanted within or inserted into a body.

According to another aspect of the invention there is provided a method of promoting maturation of osteoblasts (for example, human osteoblasts such as MG63 cells) comprising contacting the osteoblasts with FHBP and calcitriol (D3), or a method of promoting formation of osteoblasts from marrow-derived stromal cells comprising contacting the cells with FHBP.

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”, mean “including but not limited to” and do not exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects. Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

Brief Description of Figures

Embodiments of the invention will now be described, by way of example only, with reference to Figures 1-8 in which:

Figure 1 is a summary of the chemistry employed to covalently tether 1-oleoyl LPA to titanium; A shows details of the actual mechanisms for each of the different routes taken to modify titanium, in which use of the thermal initiator, azobisisobutyronitrile (AIBN), enabled 1-oleoyl LPA attachment to the metal surface; B is a schematic representation of the initial four routes chosen to functionalise titanium with 1-oleoyl LPA;

Figure 2 shows scanning electron micrographs of open porous Ti foam, A at 35x magnification, B at 50x magnification (depicting the clear porosity of the open structure), C at 170x magnification, D 650x magnification with closer inspection closer to one of the channels of the porous Ti sample;

Figure 3 shows that the LPA3 selective antagonist, FHBP, synergistically co-operates with D3 to promote human osteoblast maturation, promotion of formation of mature osteoblasts by selected agents being assessed through determining alkaline phosphatase (ALP) activity;

Figure 4 provides physiochemical evidence for the successful covalent conjugation of LPA to titanium using Time of Flight Secondary Ion Mass Spectrometry (ToF SIMS) – A: the LPA phosphate head group produced characteristic peaks at 63 and 79 m/z corresponding to PO_2^- and PO_3^-, respectively; B: the characteristic PO_2^- and PO_3^- peaks were absent from the same spectrum for non-functionalised discs;

Figure 5 is a series of SEM images showing cell morphologies of MG63 osteoblasts seeded onto 1-oleoyl LPA functionalised Ti - the images reveal many more rounded MG63 cells associated with the 1-oleoyl functionalised Ti compared to the Piranha-treated control dics;

Figure 6 shows differential maturation responses of human osteoblasts to alternative 1-oleoyl LPA functionalisation steps for solid Ti, showing that the surface with greatest potential to promote D3-induced osteoblast maturation was generated via initial treatment with Piranha solution followed by the direct attachment of 1-oleoyl LPA after thiosilane exposure (Route 1a from Figure 1);

Figure 7 shows that recycled lipid-functionalised solid titanium is still able to support D3-induced osteoblast maturation; and

Figure 8 shows open-porous titanium foam can be lipid-functionalised to enhance human osteoblast maturation – A: both 1-oleoyl LPA and FHBP modified titanium foam serve as superior substrates for D3-induced MG63 maturation compared with unmodified metal; B: scanning electron micrograph evidence for osteoblast adhesion to the titanium foam.

Examples

Materials & Methods

Lipid functionalisation of orthopaedic grade titanium

Solid titanium discs (12.7 mm diameter and 2.5 mm thickness) were a generous gift from DePuy (Blackpool, UK) whereas Ti foam discs (12mm diameter and 2mm thickness) were provided by Corin (Cirencester, UK). These latter samples had a porosity of 514mg/mm^3 +/- 66mg/mm^3 (as sourced from a random batch of 12 samples). A summary of some biomechanical and surface roughness data (as provided by Corin) for these porous samples are provided (Table 1).

Table 1

Parameter	Units	Mean	Standard deviation
Static shear strength	PSI	5314.9	609.3
Static tensile strength	PSI	7653.9	1403.8
Shear fatigue strength	PSI	4333.8	800.4
Modulus	mPA	4986	1158.7
Surface roughness	Ra µin	9531	23.86

Unless stated otherwise, all reagents were of analytical grade from Sigma-Aldrich (Poole, UK). Stocks of 1-oleoyl LPA (Enzo Life Sciences, Exeter, UK) and FHBP (tebu-bio, Peterborough, UK) were prepared in 1:1 ethanol:tissue culture grade water to a final concentration of 10mM and stored at -20°C. Likewise, stocks of D3 (100µM) were prepared in ethanol and stored at -20°C. Piranha solution was prepared by combining equal volumes (20ml) of ice-cold concentrated sulphuric acid and 30% (w/v) hydrogen peroxide. Once mixed (~10 minutes) the resultant solution was

allowed to come to room temperature before use. The covalent attachment of either 1-oleoyl LPA or FHBP involved a multi-step process. For the purposes of clarification, a summary of the chemistry of Ti functionalisation by 1-oleoyl LPA is presented (Figures 1A & 1B). In Route 1 Ti samples were treated with Piranha solution under gentle stirring for 2 hours before being washed extensively with deionised water followed by drying in desiccators. Route 1a involved the addition of a linker molecule, (3-mercaptopropyl) trimethoxysilane (MPTMS, 1ml) to batches of 8 discs immersed in 50ml anhydrous toluene. Attachment of the linker took place over 24 hours after which the discs were recovered, rinsed in dry toluene and dried prior to storage in desiccators. An alternative to MPTMS was also explored; in this instance (Route 1b) the reagent used in the functionalisation process involved the addition of vinyl trimethoxysilane (VTMS, 1ml) to Ti discs, again in anhydrous toluene (50ml) and reacted with the discs over a 24 hour period. The discs were recovered, rinsed in dry toluene before re-immersion in the same solvent (50ml) supplemented with 1,8 octanedithiol (1ml) followed by the radical initiator azobisisobutyronitrile (AIBN, 25mg). Discs were heated at 60°C and left overnight. After the treatment period the discs were recovered, rinsed in anhydrous toluene, dried and stored in desiccators.

In the final step for both routes, 100μl of the 1-oleoyl LPA or FHBP stock was added to 8 prepared Ti samples in dry toluene (50ml). Azobisisobutyronitrile (AIBN, 25mg), was added and the resultant mixture heated to 60°C and left overnight.

In this regard the influence of direct versus indirect attachment of 1-oleoyl LPA/FHBP to Ti on the osteoblast response to D3 could be explored. The functionalised Ti discs were recovered, given a triple rinse in anhydrous toluene and dried prior to physicochemical characterisation and human osteoblast exposure.

A second method for surface functionalisation for lipid attachment to Ti was also examined (Route 2). In this particular instance, untreated discs were placed in hot (60°C) concentrated NaOH (10M) and then subsequently treated in a similar manner as Route 1.

Physicochemical characterisation of lipid-functionalised Ti

Titanium foams were processed for SEM (Figure 2) as described previously (Mansell *et al.* (2010) Biomaterials vol. 31 p 199-206). To test for successful lipid modification

of Ti the samples were processed for Time-of-Flight (ToF) secondary ion mass spectrometry (SIMS) of the top 1-2 atomic layers and the spectra recorded using a IX23LS ToF SIMS instrument (Vacuum Generators) with a focussed gallium ion gun and Poschenreider energy analyser. SIMS analyses were performed on the samples under vacuum ($5x10^{-9}$ mBar) with a 1nA current gallium ion beam (20keV energy) with a pulse length of 30ns and 10kHz repetition rate. For acquisition 1600,000 pulses per spectrum over 0.3mm square area were obtained for each sample.

A Thermo Fisher Scientific (East Grinstead, UK) Escascope equipped with a dual anode X-ray source (AlK_{α} 1486.6eV and MgK_{α} 1253,6eV) was used for XPS analysis. Solid samples were analysed under high vacuum ($<5x10^{-8}$ mbar) with AlK_{α} radiation at 270W (13.5kV; 20mA). Following the acquisition of survey spectra over a wide binding energy range, the C1s, N1s, O2s, P2p, S2p, Al2p, Si2p, V2p, Ti2p spectral regions were then scanned at a higher energy resolution such that valence state determinations could be made for each element. High-resolution scans were acquired using 30eV pass energy and 200ms dwell times. Data analysis was carried out using Pisces software (Dayta Systems, Bristol, UK) with binding energy values of the recorded lines referenced to the adventitious hydrocarbons C1s peak at 284.8eV.

Contact angles were obtained for solid samples using a Krüss Drop Shape Analysis system (DSA 10 MK2) with Drop Shape Analysis software for Windows, version 1.90.0.14, calibrated to sessile drop measurements. The contact angle was an average of both right and left contact angles for each measurement. Contact angles were measured using water (between 5-7μl) with three measurements taken from the surface of each sample (four samples for each of the different surface finishes). Although humidity was not recorded, experiments were carried out in a dedicated and contained air-conditioned laboratory.

Application of human osteoblasts to lipid-functionalised Ti

Human osteoblast-like cells (MG63) were cultured in conventional tissue culture flasks (250 mL, Greiner) in a humidified atmosphere at 37°C and 5% CO_2. Although osteosarcoma-derived, MG63 cells exhibit features in common with human osteoblast precursors or poorly differentiated osteoblasts. Specifically, these cells produce type I collagen with no or low basal osteocalcin (OC) and alkaline phosphatase (ALP),

respectively. However, when MG63 are treated with D3, both OC and ALP increase which are features of the osteoblast phenotype (Clover & Gonwen (1994) Bone vol. 15 p 585-591). Consequently, the application of these cells to assess the potential pro-maturation effects of novel biomaterials is entirely appropriate.

5 Cells were grown to confluence in DMEM/F12 nutrient mix (Gibco, Paisley, Scotland) supplemented with sodium pyruvate (1 mM final concentration), L-glutamine (4 mM), streptomycin (100 ng/mL), penicillin (0.1 units/mL) and 10% v/v fetal calf serum (Gibco, Paisley, Scotland). The growth media (500 mL final volume) was also supplemented with 5 mL of a 100 X stock of non-essential amino acids.
10 Once confluent, MG63s were subsequently dispensed into blank 24-well plates (Greiner, Frickenhausen, Germany) or plates containing either control (Piranha/NaOH treated) or 1-oleoyl LPA/FHBP-modified titanium discs. In each case wells were seeded with 1 mL of a 4 X 10^4 cells/mL suspension (as assessed by haemocytometry) that was spiked with D3 to a final concentration of 100nM. For the porous Ti samples
15 cells were seeded in the same growth medium but devoid of phenol red to eliminate any interference during the alkaline phosphatase (ALP) assay described below. Cells were then cultured for 3 days, the media removed and the cells processed for total ALP activity to ascertain the extent of cellular maturation. Having identified which of the functionalisation steps was the best at supporting D3-induced osteoblast
20 maturation all subsequent studies focussed on this particular surface modification (Route 1a).

In a separate study the potential of recycling the lipid-functionalised discs was explored to ascertain whether the modified metal was still able to support an osteoblast maturation response. Briefly, discs that had already been seeded with cells
25 and processed for total ALP activity were recovered, rinsed under running tap water and scrubbed with a toothbrush to remove any remaining cellular debris. Once rinsed the samples were immersed in 70% aqueous ethanol and left for 2-3 minutes before being rinsed several times with sterile PBS. Washed discs were then placed into clean, multi-well plates, rinsed with DMEM/F12 nutrient mix and subsequently seeded with
30 MG63 cells as described above. A second repeat of this step was performed to explore how three independent uses of the same covalently functionalised discs could support the maturation of MG63 osteoblasts to D3.

Cell proliferation

An assessment of cell number was performed using a combination of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo phenyl)-2H-tetrazolium, innersalt (MTS, Promega, UK) and the electron coupling reagent phenazine methosulphate (PMS). Each compound was prepared separately in pre-warmed (37°C) phenol red free DMEM/F12, 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. The resultant mixture was filtered via 0.2 μm and 0.1 mL dispensed into each well. A blank consisted of media alone (0.5 mL) plus 0.1 mL of the MTS/PMS reagent mixture. Plates were left for 45 min under conventional culturing conditions. Once incubated, samples (0.1 mL) from each well were dispensed onto a 96-well microtitre plate and the absorbances at 492 nm read using a multiplate reader. Plates were staggered to ensure that all samples were recovered for 96-well plating within 5 min to minimise any error introduced during the formation of further formazan product by the cell monolayer. Plotting the absorbances against known cell number, as assessed initially using haemocytometry, enabled extrapolation of cell numbers for the experiments described herein.

Total ALP activity

An assessment of ALP activity is reliably measured by the generation of p-nitrophenol (p-NP) from p-nitrophenylphosphate (p-NPP) under alkaline conditions. The treatment of cells to quantify ALP activity was similar to that described previously (Gidley *et al.* (2006) Prost. and other Lipid Med. vol. 80 p 46-61). Briefly, the remaining MTS/PMS reagent was removed and the monolayers incubated for a further 15 min in fresh phenol red free DMEM/F12 to flush out the residual formazan. Following this incubation period the medium was removed and the monolayers lysed with 0.1 mL of 25 mM sodium carbonate (pH10.3), 0.1% (v/v) Triton X-100. After 2 min each well was treated with 0.2 mL of 15 mM p-NPP (di-tris salt, Sigma, UK) in 250 mM sodium carbonate (pH 10.3), 1mM $MgCl_2$. Lysates were then left under conventional cell culturing conditions for 1 h. After the incubation period, 0.1 mL aliquots were transferred to 96-well microtitre plates and the absorbance read at 405 nm. An ascending series of p-NP (25–500 μM) prepared in the incubation buffer enabled quantification of product formation. To ensure that the ALP activity was

associated for cells only attached to Ti and not to the surrounding plastic, the sample discs were transferred to clean multiwell plates and then processed for ALP activity.

Statistical analysis

Unless stated otherwise, all experiments described above were performed three times and all data were subject to an unpaired, two-tailed t-test assuming unequal variance. A p value of <0.05 was deemed to be statistically significant between groups.

Rat tail implant model

Female Wistar rats (Crl(Wi)Br, Charles River; 250-370g) from the breeding colony of Semmelweis University are kept in light controlled, air-conditions rooms and fed *ad libitum*. The animals are divided into two groups, group 1 being control increased surface area implants and group 2 being phospholipid-functionalised increased surface area implants. In both cases, $n = 20$.

Cylindrical implants of 2.1mm x 4.5mm are prepared from titanium, either untreated (control) or phospholipid-functionalised, for example with 1-oleoyl LPA or FHBP.

Rats are anaesthetised with sodium pentobarbital (Nembutal 40 mg/kg body weight (b.w.), by intraperitoneal (i.p.) injection.

The rat tail is desinfected and ligatured to control bleeding during surgery. A circumferential incision is made distal to the level of the caudal C4-C5 intervertebral space. The skin is retracted and the tail is cut between the two vertebrae under sterile conditions. On the exposed cut intervertebral surface of the C4 vertebra a central 1 mm diameter and 5 mm deep perpendicular drillhole is formed to guide the 2.1 mm drill, which corresponds to the size of titanium implant, using an electric drill. As a result a cavitary bone defect will be formed to accommodate the 2.1 mm x 3.5 mm Ti implant.

Following insertion of the implant the skin is repositioned over the implant and tightly sutured. The surgical wound is protected aseptically by a plastic film-layer (Plastubol®: methylmethacrylatbutylacrylat-butylmethacrylat, diisooctyl phthalate; Pannonpharma Ltd., Hungary).

Rats are kept in individual cages to insure appropriate hygiene and wound healing during the first two weeks following surgery. The extent of osseointegration is assessed using mechanical pull-out strength tests and results between groups 1 and 2 are compared.

5 *Larger animal implant model in sheep*

A larger animal model is based, for example, on that described by Blom *et al.* (2005) Journal of Bone and Joint Surgery (Br) vol. 87-B p 421-425. Ten skeletally mature Welsh mule ewes (body-weight approximately 47 to 76 kg) are subjected to right unilateral hemiarthroplasty. Pre-medication comprises 0.1 mg/kg IM xylazine 10 (Rompun®, Bayer PLC, Bury St Edmunds, UK) and the prophylactic antibiotic sodium cefalexin 1 mg/18 kg (Ceporex®, Schering-Plough, Welwyn Garden City, UK).

Anaesthesia is induced using 2.5 mg Midazolam (Hypnovel®, Roche, Welwyn Garden City, UK) and 2 mg/kg ketamine hydrochloride (Ketaset®, Fort Dodge 15 Animal Health Limited, Southampton, UK) and maintained after intubation with halothane, oxygen and nitrous oxide. Post-operative analgesia is provided using 0.6 mg buprenorphine (Temgesic®, Schering-Plough) twice daily.

A craniolateral approach is used and all cancellous bone is removed from the proximal femur. Femoral impaction grafting is performed with a titanium stem, either 20 untreated or phospholipid-functionalised according to the invention.

The sheep are allocated randomly to two groups each of five ewes. Group one animals receive control untreated titanium implant and group two animals receive 1-oleoyl LPA or FHBP functionalised titanium implant.

In vivo assessments are made pre-operatively and at one or more of 1, 2, 4, 6, 12 and 25 18 months after the operation.

To assess peak ground reaction forces, the sheep are walked over a piezo-electric force plate (Kistler Instruments AG, Winterthur, Switzerland) in a walkway. Twelve readings of peak ground reaction forces are taken from each hind limb. The sheep are weighed on the same piezo-electric force plate and forces normalised to body-weight.

Craniocaudal radiographs are taken and DEXA scanning is carried out according to the methods set out in Blom *et al.* (2005). Post mortem examination of retrieved specimens is also carried out according to the methods of Blom *et al.* (2005).

Results

5 *The novel LPA3 selective agonist, FHBP, synergistically co-operates with D3 to enhance MG63 maturation*

Time (24, 48 and 72 h) and dose (250-2000nM) response studies were conducted for FHBP for its ability to evoke a maturation response in human osteoblasts, either alone or in combination with 100nM D3. The findings presented clearly indicate that FHBP 10 acts synergistically with D3 to promote statistically significant time and dose dependent increases in p-NP and therefore ALP activity in MG63 cells (Figure 3). A reduction in potentcy was observed at the highest FHBP concentration; without being bound by theory, this may be caused by receptor down-regulation. The synergistic increase in p-NP, and therefore total alkaline phosphatase activity, for the cells co-15 stimulated with D3 and FHBP was independent of cell number (Table 2). These findings are similar to the previously published data for the co-treatment of MG63 cells with LPA and D3 and for cells exposed to a combination of 2(S)-OMPT and D3.

Table 2

Time point	**24hr**	**48hr**	**72hr**
Vehicle	12.7±0.9	13.1±1.1	10.1±0.6
D3 only	19.0±1.2	18.7±1.4	16.8±1.1
0.25μM FHBP	13.8±0.8	24.0±1.4	27.3±1.8
0.5μM FHBP	14.2±1.2	23.6±2.1	23.7±1.5
1.0μM FHBP	12.1±0.7	22.7±1.3	24.7±1.7
2.0μM FHBP	12.5±1.1	15.8±1.2	15.6±0.9
0.25μM FHBP & D3	71.9±4.7	151.0±8.9	225.5±21.7
0.5μM FHBP & D3	62.4±5.3	145.6±16.1	207.4±18.5
1.0μM FHBP & D3	56.4±7.1	139.2±11.5	187.6±19.1
2.0μM FHBP & D3	46.9±5.3	95.9±5.2	90.7±11.2

20 *Physiochemical evidence for the successful covalent conjugation of LPA (lipids) to titanium*

Time of Flight Secondary Ion Mass Spectrometry (ToF SIMS) was used to determine the presence of 1-oleoyl LPA on modified titanium. The 1-oleoyl LPA phosphate

head group produced characteristic peaks at 63 and 79 m/z corresponding to PO_2^- and PO_3^-, respectively. These signals were recorded from the negative ion spectrum of the functionalised discs indicating the presence of phosphate-containing 1-oleoyl LPA on the metal surface. The characteristic PO_2^- and PO_3^- signals were absent from the same
5 spectrum for non-functionalised control discs (Figure 4).

In order to provide further evidence of surface functionalisation, X-ray photoelectron spectroscopy (XPS) was used to analyse the surfaces of both functionalised and control samples. The Piranha treated control samples yielded carbon (37.7%), oxygen (35.8%) and sulphur (11.3%), whereas the silane-functionalised samples exhibited
10 carbon (49.2%), oxygen (24.3%), silicon (17.3%) and sulphur (2%). It is noteworthy that no phosphorus was detected on either of the controls. The large carbon content for the Piranha sample is possibly the result of adsorption after drying. The presence of oxygen and sulphur on the Piranha-treated control sample is a consequence of the treatment in the strongly oxidising sulphur-containing Piranha solution. This not only
15 hydroxylates the surface but also appears to deposit sulphate ions onto it. However, there is no indication of either phosphorus or silicon on the Piranha-treated control. The increase in carbon content and the presence of silicon were expected and attributed to the addition of the silane coupling agent.

Analysis of the 1-oleoyl LPA-functionalised sample showed the presence of silicon
20 (11.6%), phosphorus (0.8%) as well as an increase in carbon content (50.9%). Using calculations based on the molecular volumes of the species involved (see Appendix A), the inventors estimated that the covalent approach yielded coverage of 45.8% of the maximum theoretical coverage.

Contact angle measurements (Table 3) were used to determine the influence of
25 surface groups (i.e., whether the surface of the samples was hydrophobic or hydrophilic) and to provide further indirect proof whether successful attachment of the 1-oleoyl LPA had occurred. Four samples were measured; a control (untreated sample), a Piranha-treated sample, a silane-treated sample and a 1-oleoyl LPA-functionalised sample.

30

Table 3

Sample	Angle 1 °	Angle 2 °	Angle 3 °	Average °
Control	62.5	67.2	71.2	67.0
Piranha	34.7	35.7	36.0	35.5
Silane	122.0	117.6	119.4	119.7
Silane plus LPA	76.0	85.0	78.0	79.7

Both the Piranha and untreated control samples yielded hydrophilic surfaces, with average contact angles of 35.5° and 67.0°, respectively. The greater hydrophilicity of
5 the Piranha sample is attributed to the high degree of hydroxylation of the sample surface. An increase in contact angle was observed for the silane treated sample, with an average angle of 119.7°. As attached LPA would present a hydrophilic head group it would be expected that the contact angle would decrease from that of the silane sample, This was indeed observed, with the contact angle decreasing to an average of
10 79.9°, showing that 1-oleoyl LPA had been successfully attached to the sample.

These clear changes in surface polarity are reflected in the findings for early attachment (2 hours) and spreading (24 hours) of the MG63 cells upon solid Ti discs. The findings presented indicate persistence of more rounded morphologies for cells seeded on to LPA-functionalised surfaces compared to the Piranha controls, even after
15 24 hours (Figure 5).

Lipid-functionalised solid Ti is a better substrate for supporting D3-induced osteoblast maturation than unmodified metal

Initially the inventors screened solid Ti discs that had been subject to several different 1-oleoyl LPA functionalisation treatment regimens to ascertain which route yielded
20 the most robust maturation response for MG63 cells. The data (Figure 6) indicate that Route 1a (i.e., the LPA-functionalised discs exposed to initial Piranha solution treatment followed by the direct attachment of the lipid) produced a modified surface with the greatest (*$p<0.002$ compared to the Piranha control) potential to promote D3-induced osteoblast maturation, as supported by the greater total ALP activity. From
25 this point on all subsequent modifications of Ti used adopted this strategy of functionalisation.

In a second series of experiments (Figure 7) Ti discs were processed using Route 1a for attachment of either 1-oleoyl LPA or the LPA3 selective agonist, FHBP and seeded with MG63 cells in the presence of 100nM D3. After 72 hours of culture the discs were treated to assess total ALP activity. The data clearly indicate that "first use" discs coated with either 1-oleoyl LPA (*$p<0.001$) or FHBP (**$p<0.001$) support the maturation of human osteoblasts compared to control discs. The discs were subsequently recovered from the wells and subsequently re-seeded with cells to ascertain whether these recycled discs still exhibited bioactivity. Although total ALP activity was less for cells associated with "second use" discs, the data do support that covalent attachment of 1-oleoyl LPA is effective. Surprisingly, recycled samples do exhibit the greater capacity to support human osteoblast maturation compared to controls. Even more surprisingly, similar results were obtained for "third use" discs, where the maturation response of cells to FHBP coating is superior ($^{\S}p<0.001$) to that of 1-oleoyl LPA. Collectively, the data support the robust attachment of biologically active LPA receptor agonists to orthopaedic grade solid titanium.

Evidence for the successful LPA/FHBP-functionalisation of open, porous Ti foam

Both 1-oleoyl LPA and the selective LPA3 receptor antagonist, FHBP, were immobilised onto porous Ti using Route 1a (i.e., initial Piranha solution treatment followed by the direct attachment of these lysophospholipids to the metal surface). The data depicted for total ALP activity provide a clear indication that both 1-oleoyl LPA and FHBP modified Ti foams serve as superior substrates for D3-induced MG63 maturation compared to unmodified metal (*$p<0.001$, Figure 8A). Evidence for osteoblast adhesion to the Ti foam is also presented (Figure 8B) as a scanning electron micrograph image.

Discussion

Over the past few decades a concerted global effort has ensued to enhance the performance and longevity of bone biomaterials through surface modifications. With regard to biological agents, the molecules chosen are invariably proteins/peptides that either influence osteoblast adhesion (typically molecules that bind to cell surface integrins), or they are growth factors known to drive osteogenesis. With reference to cell adhesion molecules, researchers have examined the potential of Arg-Gly-Asp (RGD) containing peptide coatings to aid osteoblast attachment and mimic the

signalling incurred via the collagenous matrix they secrete. However, the *in vivo* evidence indicates that these material modifications offer, at best, modest improvements on the process of osseointegration. (Lutz *et al.* (2010) Clin. Oral Implants Res. vol. 21 p 726-734). Given the likely costs generated to fabricate such materials they are unlikely to find use as functional adjuncts for bone repair and/or regeneration.

The focus for the use of growth factors primarily pertains to non-covalent interactions of large polypeptides/proteins with bone biomaterials, for example, transforming growth factor-β, platelet-derived growth factor, insulin-like growth factors and, most significantly, bone morphogenic proteins (BMPs). Indeed, the steps taken to explore the benefits of, for example, BMPs, in a biomaterials setting have focussed on (non-covalent) adsorption techniques, e.g., incorporation into the actual Ti oxide layer (Wikesjö *et al.* (2008) Clin. Periodontol. vol. 35 p 992-1000), carried within mineral phase coatings (Liu *et al.* (2005) Bone vol. 36 p 745-757), dispersal via biodegradable scaffolds such as collagen sponges (Boyne *et al.* (2004) Implant. Dent. vol. 13 p 180-184) or delivery using type I collagen telopeptides (Bessho *et al.* (1999) Clin. Oral Implants Res. vol. 10 p 212-218). However, the high cost of these innovative strategies using recombinant BMPs likely precludes widescale application. Also, their large size would be predicted to pose potential attachment and delivery concerns at or around biomaterials as well as possible sterical constraints in targeting cell surface receptors. Importantly, at the time of the present study, there have been no reports describing the successful, covalent attachment of a growth factor known to influence human osteoblast formation and/or maturation at a titanium surface.

Given the potential problems posed by large biomacromolecules, the inventors considered far smaller growth factors that might be more amenable to covalent attachment to biomaterial surfaces. The rationale behind this approach was to preserve (or even enhance) their signalling capabilities and reduce the potential of steric hindrance to their cell surface receptor proteins. One particularly attractive candidate for titanium bio-functionalisation is the small lipid 1-oleoyl LPA and certain LPA receptor agonists, e.g., OMPT and FHBP. Of particular significance to bone formation and, therefore, the potential to secure a mechanically sound mineralised matrix, is the discovery that 1-oleoyl LPA/OMPT (and now FHBP), can synergistically co-operate

with D3, to promote human osteoblast maturation at the titanium surface. A large increase in total ALP, an enzyme essential for bone collagen calcification (Whyte (2010) Ann. N.Y. Acad. Sci. vol. 1192 p 190-200), is a reliable indicator of osteoblast maturation. It was this striking co-operative influence of D3 and 1-oleoyl LPA on
5 cellular maturation (Gidley *et al.* (2006) Prost. And other Lipid Med. vol. 80 p 46-61) that led the inventors to focus on 1-oleoyl LPA and related lipids for their covalent attachment to Ti.

A covalent method of attachment was deliberately sought, primarily to ensure that the lipids are more likely to remain bound when subject to the forces encountered during
10 implant insertion and physical activity post-operatively. In order to ensure that the phosphate head group of the 1-oleoyl LPA/FHBP would be accessible to cell surface receptors, attention was focussed on the alkene moiety for covalent modification. Appropriate preparation of the Ti surface enables the use of silane-based functionalisation to present either a thiol reactive group for direct radical-mediated
15 addition to the LPA double bond, or, as an alternative route, functionalisation with an alkene-modified silane for function reaction with a dithiol (see Figure 1). The possible drawback of reaction of the dithiol species reacting twice with surface-anchored alkene groups (and thus not being available to react with LPA in a following step) was confirmed by the lower activity by discs modified by this route.

20 Clear evidence is provided herein for the successful functionalisation of both solid and porous Ti with either LPA or the LPA receptor agonist, FHBP. These novel biomaterials successfully co-operated with D3 to secure human osteoblast maturation, as supported by the marked, synergistic increases in total ALP activity. Having initially seeded MG63 cells on to control and lipid-modified Ti the samples were
25 recovered, rinsed under running tap water, cleaned and reused for both a second and third time to ascertain whether "recycling" samples still yielded a bioactive surface. The primary purpose of this series of experiments was to provide evidence that the 1-oleoyl LPA/FHBP could remain attached with recycling of the Ti samples. Although Ti prostheses are not "recycled" in the clinical setting, it is worth noting that, during a
30 process such as arthroplasty, devices which do not fit at the first attempt are recovered, irrigated and re-positioned. Therefore, evidence to indicate that additional

washings/irrigations do not disrupt the bioactive surface is important in the overall development of a covalently modified bioactive Ti device.

Although there is a clear pattern of loss of functionalisation with reuse, the samples were still much better than control metals at stimulating MG63 maturation. It is likely, therefore, that the methodology adopted here in generating lipid-functionalised Ti could withstand the rigors of arthroplasty. Recently, it has emerged that human, bone marrow-derived, stromal cells (hBMSCs) express LPA receptors and that exposure of these cells to 1-oleoyl LPA can lead to osteoblast formation and resultant matrix mineralisation (Liu *et al.* (2010) J. Cell. Biochem. vol. 109 p 794-800). The finding that hBMSCs can differentiate into osteoblasts in response to 1-oleoyl LPA would indicate that 1-oleoyl LPA-functionalised Ti may indeed be a choice substrate to achieving enhanced osteogenesis at the implant surface.

Conclusions

The research presented is the first to describe the fabrication of lipid-functionalised Ti surfaces that are better suited to support human osteoblast maturation compared to unmodified metal. These studies provide an important first step in realising the development of superior, next-generation, functional biomaterials for improving host cell responses conducive to enhancing osseointegration, performance and longevity.

Appendix A

All molecular radius measurements were calculated using Spartan version '06.

Surface area of one face of titanium disk – 1.27E-04m^2

Molecule	Radius (Å)	Surface area (m^2)
Silane coupling agent	4.39	6.04E-19
1-oleoyl LPA	5.49	9.47E-19

5 Ratio of 1-oleoyl LPA and silane surface areas

=6.04/9.47 = 0.638 = 1 LPA : 1.56 silane (rounded to 2 for calculation below)

XPS data shows 3.49% of surface is sulphide.

The LPA:silane ratio can then be used to obtain the theoretical maximum % of phosphorus (and therefore LPA) than can bond to the surface, using information

10 obtained from the XPS:

3.49/2 x 100 = 1.745%

XPS data shows that a functionalised disc contains 0.8% phosphorus. Therefore, the yield for LPA is:

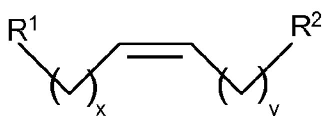
0.8/1.745 x 100 = 45.8%

15

Claims

1. An orthopaedic material comprising a biocompatible material and at least one organic LPA receptor agonist compound, the compound comprising at least one inter-carbon double bond and being covalently bound to a surface of the
5 biocompatible material via a carbon in the double bond.

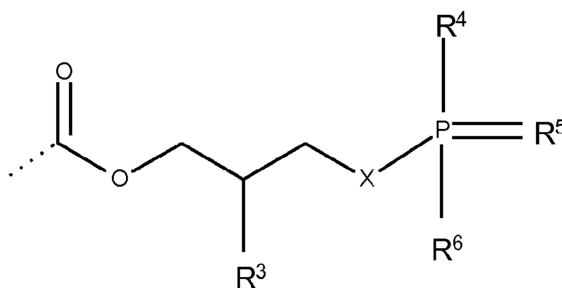
2. Material according to claim 1 wherein the LPA receptor agonist compound comprises the motif of Formula I:



Formula I

where R^1=Formula II, R^2=CH_3 and x=y=7.

10 3. Material according to claim 1 or 2 wherein the LPA receptor agonist compound comprises the motif of Formula II:



Formula II

where R^3 is OH or OR, R^4 is OH, Na^+O^- or OR, R^5 is O or S, R^6 is OH, Na^+O^- or OR and X is O or CHY, where Y is halide and R within OR is alkyl.

15 4. Material according to claim 1, 2 or 3 wherein the LPA receptor agonist is 1-oleoyl LPA or 1-fluoro-3-hydroxy-4-butyl-1-phosphonate (FHBP) or 1-oleoyl-2-methyl-*sn*-glycero-3-phosphothionate (OMPT) or lysophosphatidyl-methanol (LPM).

5. Material according to any preceding claim wherein the biocompatible material comprises titanium, a titanium alloy, stainless steel, tantalum, a tantalum alloy, polyethylene, hydroxyapatite, a natural polymer material or a saturated aliphatic polymer material.

5 6. Material according to claim 5 wherein the biocompatible material comprises titanium.

7. Material according to any preceding claim wherein the LPA receptor agonist compound is 1-oleoyl LPA.

8. Material according to any of claims 1-6 wherein the LPA receptor agonist
10 compound is FHBP.

9. Material according to any of claims 1-6 wherein the LPA receptor agonist compound is OMPT or LPM.

10. Material according to any preceding claim characterised in that it promotes calcitriol-induced osteoblast maturation as compared to the maturation
15 obtained using the biocompatible material which does not have an LPA receptor agonist compound covalently bound to a surface thereof.

11. Material according to claim 10 wherein the osteoblast is a human osteoblast.

12. Material according to any preceding claim characterised in that it promotes formation of osteoblasts from marrow-derived stromal cells as compared to
20 the formation obtained using the biocompatible material which does not have an LPA receptor agonist compound covalently bound to a surface thereof.

13. Material according to any preceding claim which is formed as an orthopaedic implant device or portion thereof.

14. Method of obtaining a item according to any preceding claim comprising the
25 steps of:
    a) immersing or coating a biocompatible material item in Piranha solution at room temperature, or in 10M NaOH at elevated temperature;
    b) washing the item with deionised water and drying the item;

c) immersing or coating the item in anhydrous organic solvent;
d) adding a linker molecule;
e) rinsing the item in anhydrous organic solvent and drying the item;
f) adding an organic LPA receptor agonist compound stock solution in anhydrous organic solvent;
g) adding a radical initiator and exposing the item to a reaction initiator ;
h) rinsing the item in anhydrous organic solvent;
i) drying the item and optionally storing before use;

wherein step (d) comprises either the step of adding a reactive intermediate and proceeding to step (e), or the steps of adding a difunctional linker, rinsing the item in anhydrous organic solvent, adding anhydrous organic solvent supplemented with 1,8 octanedithiol, adding a radical initiator and exposing the item to a reaction initiator and proceeding to step (e).

15. Method according to claim 14 comprising the steps of:
a) immersing or coating a titanium biocompatible material item in Piranha solution at room temperature, or in 10M NaOH at about 60°C;
b) washing the item with deionised water and drying the item;
c) immersing or coating the item in anhydrous toluene;
d) adding a linker molecule;
e) rinsing the item in anhydrous toluene and drying the item;
f) adding an organic LPA receptor agonist compound stock solution in anhydrous toluene;
g) adding azobisisobutyronitrile (AIBN) and heating at about 60°C;
h) rinsing the item in anhydrous toluene;
i) drying the item and optionally storing before use;

wherein step (d) comprises either the step of adding (3-mercaptopropyl) trimethoxysilane (MPTMS) and proceeding to step (e), or the steps of adding vinyl trimethoxysilane (VTMS), rinsing the item in anhydrous toluene, adding anhydrous toluene supplemented with 1,8 octanedithiol, adding AIBN and heating at about 60°C and proceeding to step (e).

16. Method according to claim 14 or 15 wherein the organic LPA receptor agonist compound stock solution is a stock solution of a phospholipid.

17. Method according to claim 16 wherein the organic LPA receptor agonist compound stock solution is a 1-oleoyl LPA stock solution or FHBP stock solution or OMPT stock solution or LPM stock solution.

18. Method according to claim 17 wherein the organic LPA receptor agonist compound stock solution is 1-oleoyl LPA stock solution, formed by preparing a solution of LPA at a concentration of about 10mM in about 1:1 ethanol:tissue culture grade water.

19. Method according to claim 17 wherein the organic LPA receptor agonist compound stock solution is FHBP stock solution, formed by preparing a solution of FHBP at a concentration of about 10mM in about 1:1 ethanol:tissue culture grade water.

20. Method according to any of claims 14-19 wherein step (d) comprises the step of adding (3-mercaptopropyl) trimethoxysilane (MPTMS) and proceeding to step (e).

21. Method according to any of claims 14-20 wherein the immersion or coating in step (a) continues for about 2 hours.

22. Method according to any of claims 14-21 wherein, in step (d), the item is immersed or coated in MPTMS or VTMS for about 24 hours.

23. Method according to any of claims 14-22 wherein, in step (d) and/or step (g), the item is immersed or coated in AIBN and heated at about 60°C for a period of 8-15 hours.

24. Method according to any of claims 14-23 wherein the Piranha solution is formed by combining equal volumes of ice-cold concentrated sulphuric acid and about 30% (w/v) hydrogen peroxide, mixing for about 10 minutes and allowing the mixture to come to room temperature prior to use.

25. Orthopaedic material obtained by a method of any of claims 14-24.

26. Material according to claim 13 or 25 for use in a method of implanting a surgical device into a human or animal body.

27. Method of implanting a surgical device into a human or animal body comprising implanting a material according to claim 13 or 25.

28. Method of promoting maturation of osteoblasts on a surface comprising forming the surface from an orthopaedic material according to any of claims 1-13 or 25 and contacting the surface with a sample of osteoblast cells.

29. Method according to claim 28 comprising contacting the surface and osteoblasts with calcitriol (D3).

30. Method of promoting formation of esteoblasts on a surface comprising forming the surface from an orthopaedic material according to any of claims 1-13 or 25 and contacting the surface with a sample of marrow-derived stromal cells.

31. Method of promoting maturation of osteoblasts comprising contacting the osteoblasts with FHBP and calcitriol (D3).

32. Method of promoting formation of osteoblasts comprising contacting a sample of marrow-derived stromal cells with FHBP.

Figure 1A

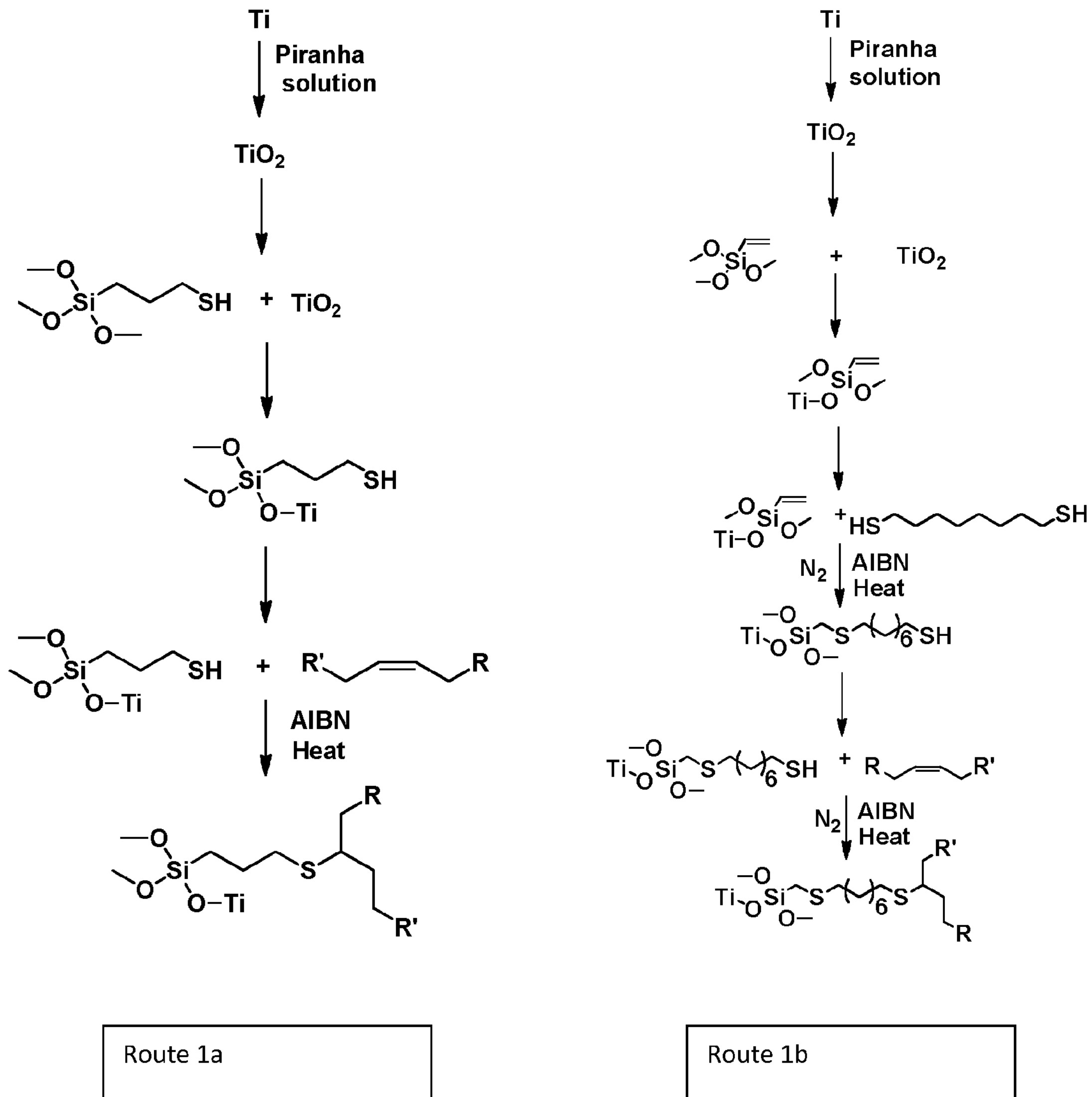
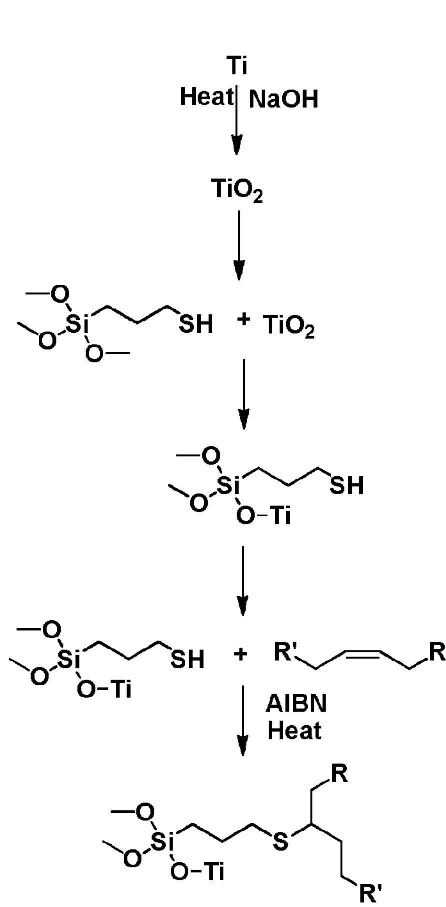
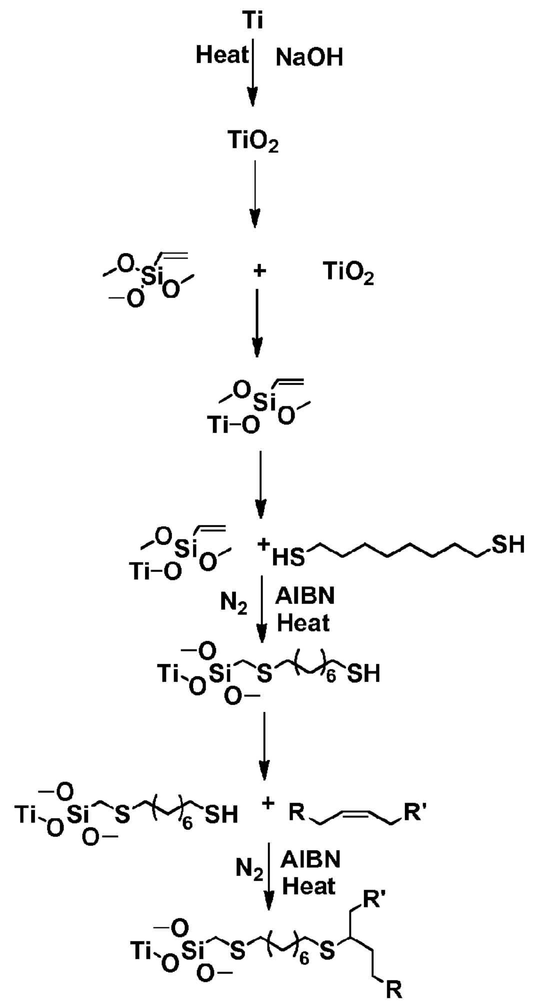


Figure 1A (continued)

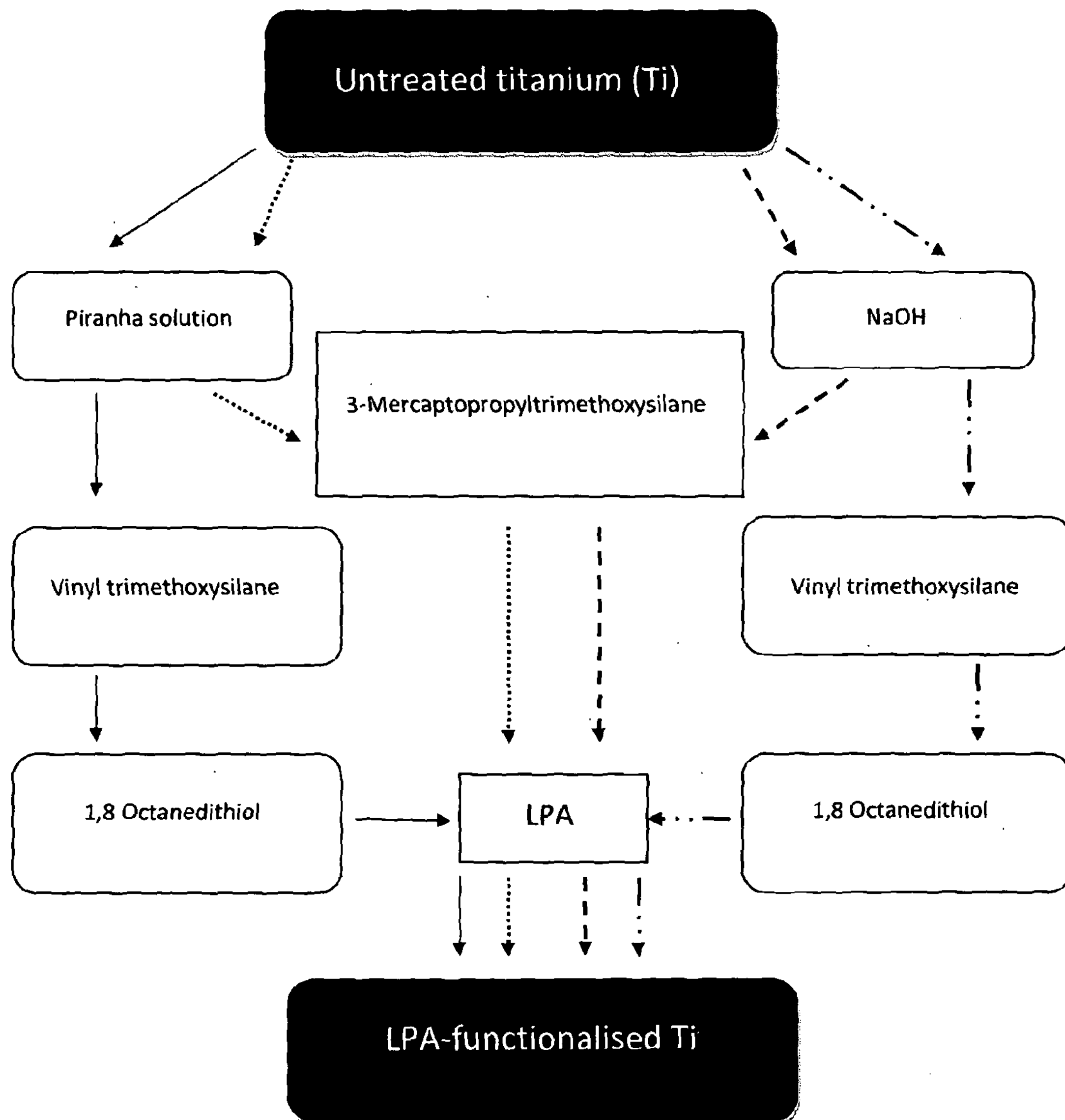




Route 2a

Route 2b

Figure 1B



Legend:

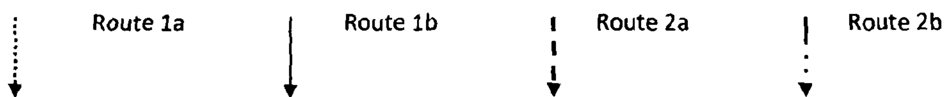
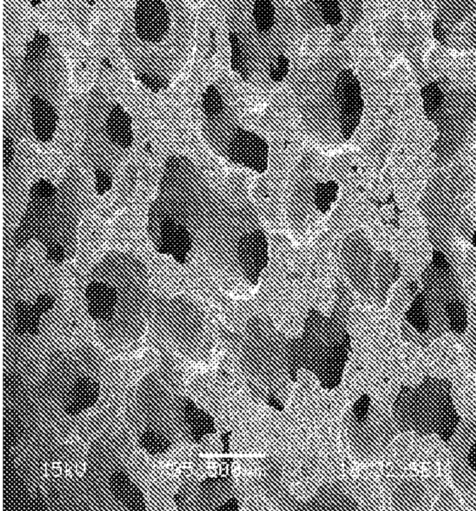
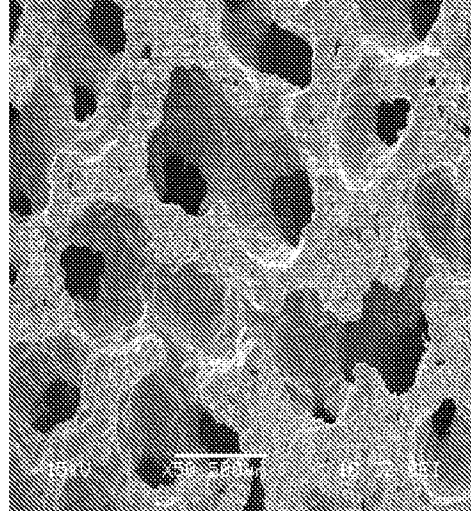


Figure 2

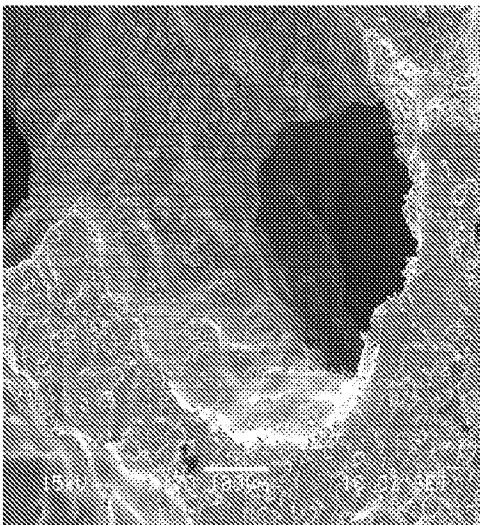
A



B



C



D

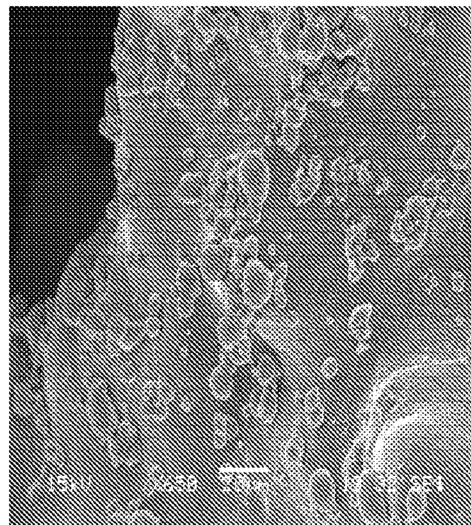


Figure 3

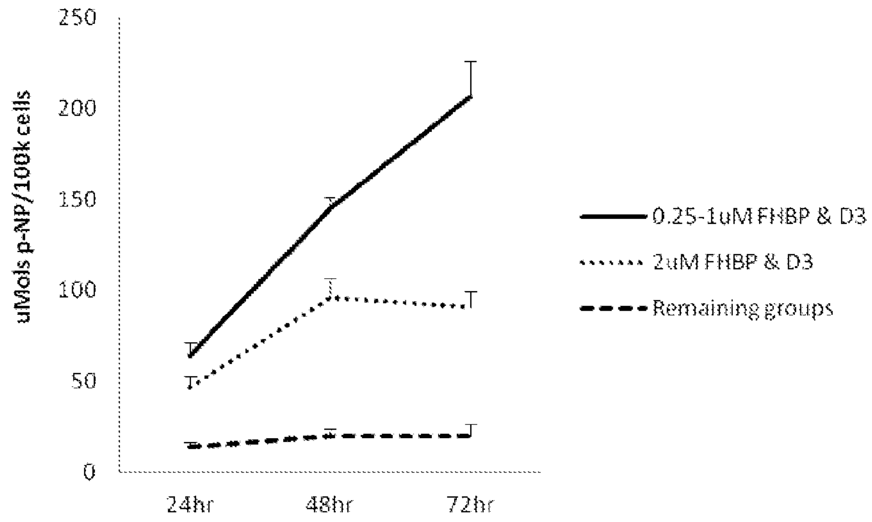


Figure 4A

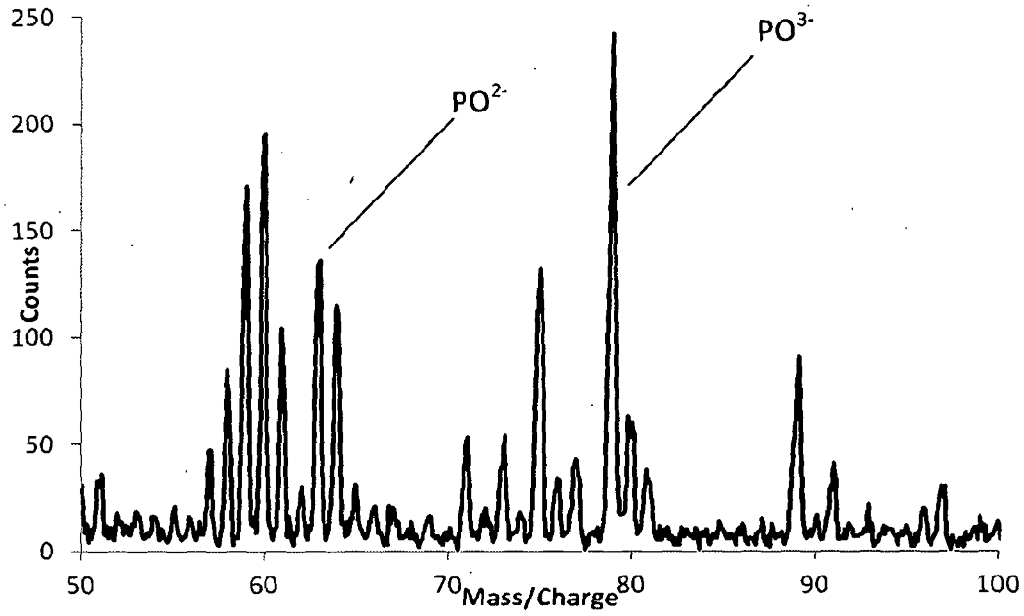


Figure 4B

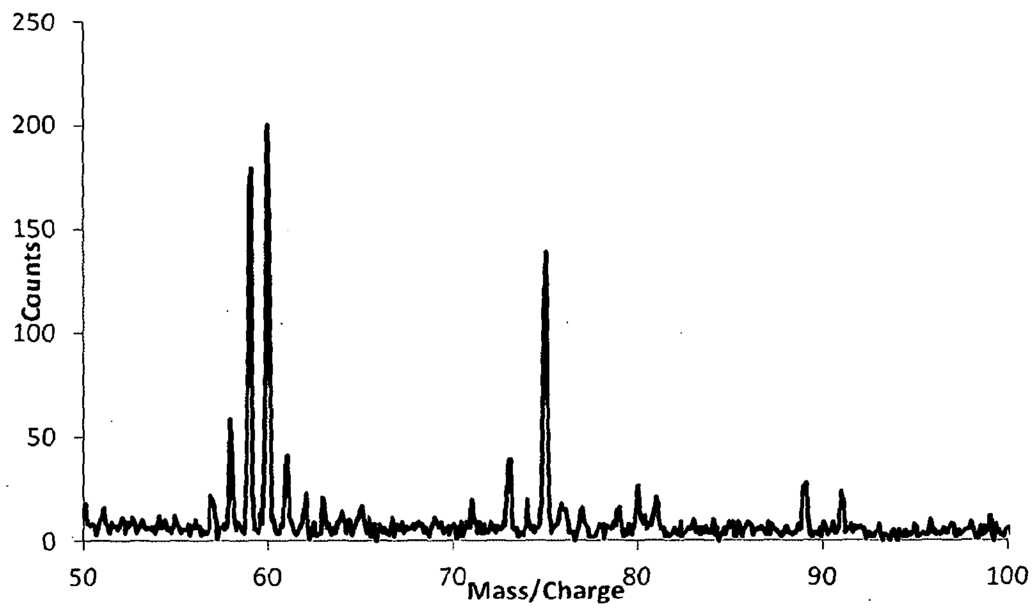


Figure 5

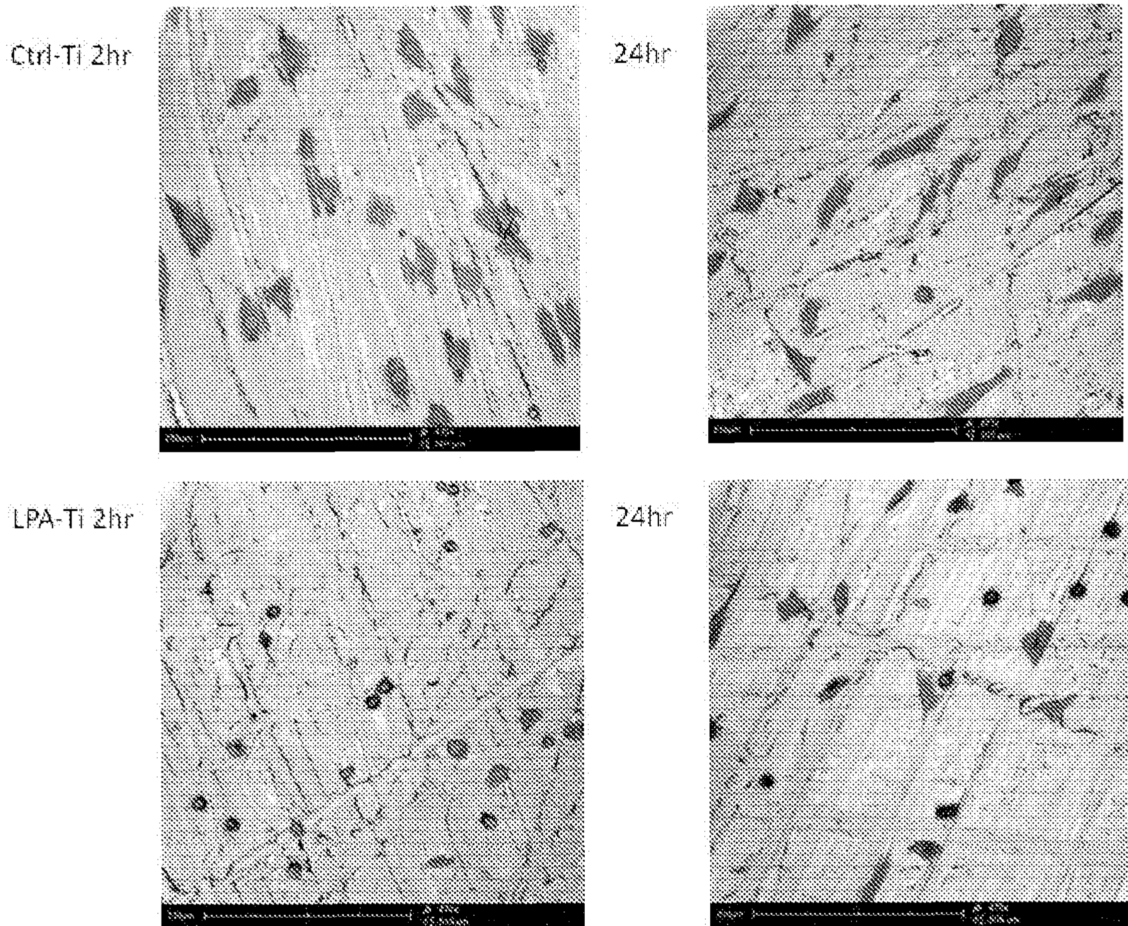


Figure 6

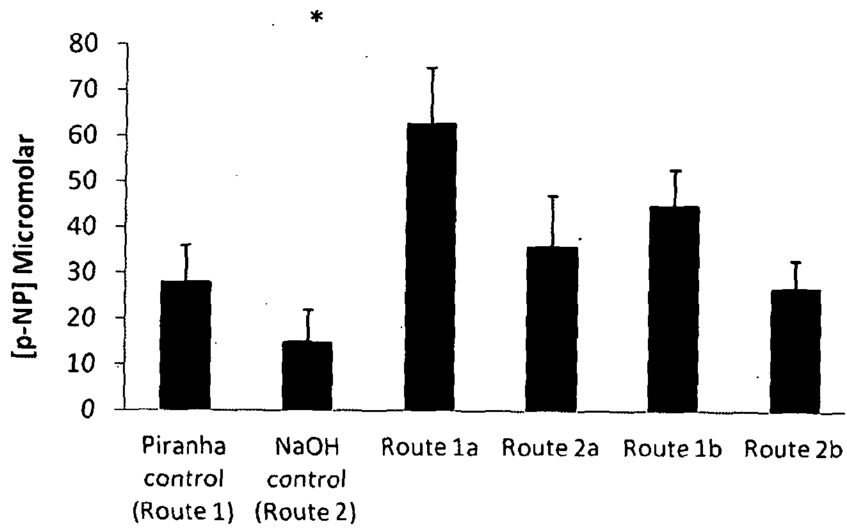


Figure 7

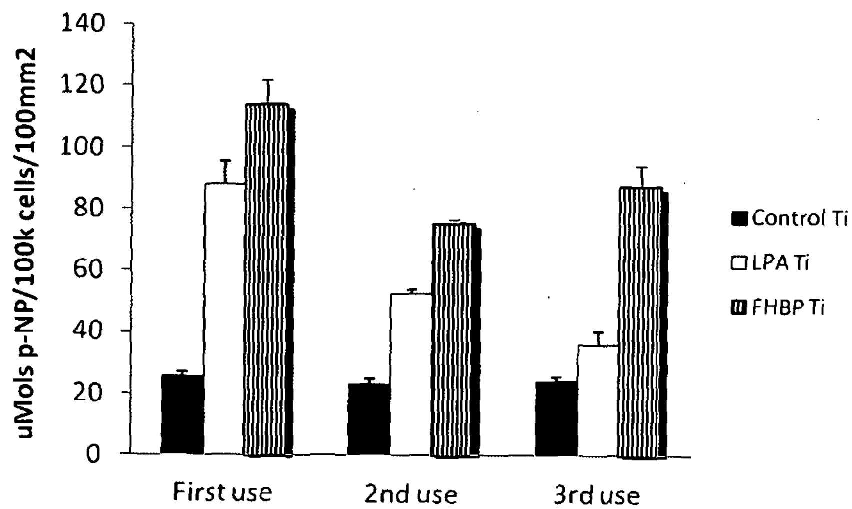
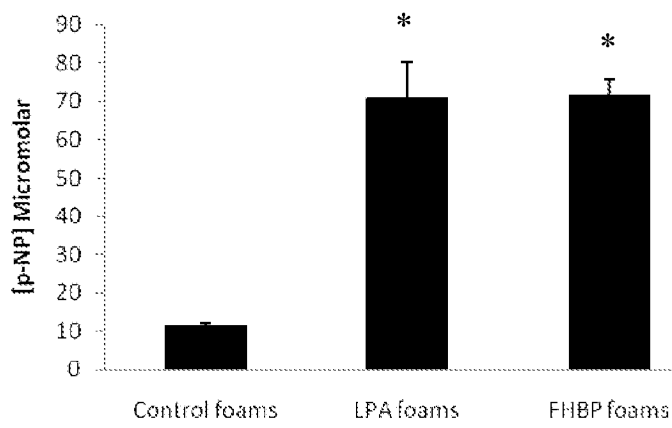
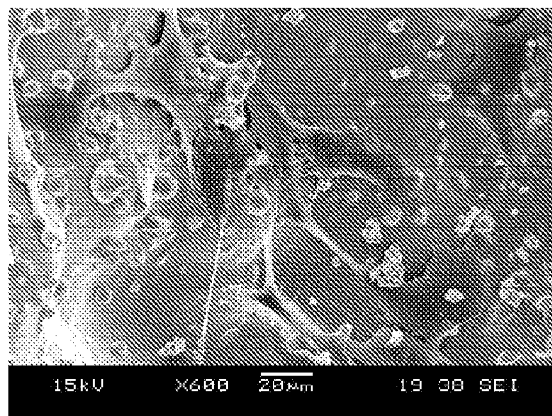


Figure 8

A



B



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/051110

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61L27/06 A61L27/50 A61L27/54
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/123765 A1 (ONG JOO L [US] ET AL) 9 June 2005 (2005-06-09)	1,2,5,6, 10-16, 20-32
Y	paragraphs [0086] - [0107] figures 5,6	3,4,7-9, 17-19

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2012	02/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	López García, Mónica

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/051110

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MANSELL J P ET AL: "The synergistic effects of lysophosphatidic acid receptor agonists and calcitriol on MG63 osteoblast maturation at titanium and hydroxyapatite surfaces", BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 31, no. 2, 1 January 2010 (2010-01-01), pages 199-206, XP026745990, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2009.09.035 [retrieved on 2009-09-30] abstract page 203, right-hand column, paragraph 2 page 204, left-hand column, paragraph 2 -----	3,4,7-9, 17-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2012/051110

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005123765 A1	09-06-2005	AU 2002359715 A1	09-07-2003
		US 2005123765 A1	09-06-2005
		WO 03053218 A2	03-07-2003