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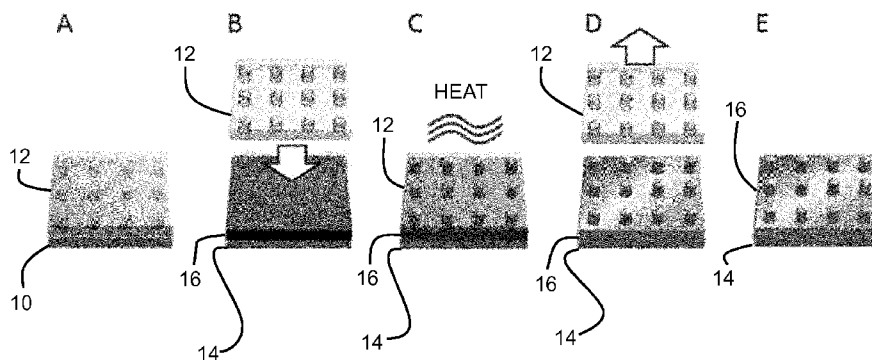
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(54) **Title:** NANOSTRUCTURED SURFACES



**Fig. 8**

(57) **Abstract:** A method of forming nanostructures on a curved surface of a substrate, such as a metallic medical implant, is disclosed. A conformable template is provided, having template nanostructures. A sol-gel comprising a patterning precursor is applied to the curved surface with the sol-gel interposed between the conformable template and the curved substrate. In this way, a patterned coating of patterning precursor on the curved substrate is formed. Next, a thermal treatment of the patterned coating is carried out in order to develop the nanostructures.

## **NANOSTRUCTURED SURFACES**

### **BACKGROUND TO THE INVENTION**

#### 5 **Field of the invention**

The present invention relates to the formation of nanostructured surfaces and to components formed with such nanostructured surfaces. In particular, although not necessarily exclusively, the present invention relates to nanopatterning of a curved  
10 surface of a substrate for use, for example, as a medical implant.

#### **Related art**

It is known that surface modification of materials at the nanometre scale can give rise to  
15 useful properties. Typically, polymeric materials have been targeted for surface modification, because the modification can be physically incorporated by embossing or imprinting.

In the case of medical implant devices however, the majority of orthopaedic devices are  
20 metal based, because polymeric materials may not have the necessary mechanical properties. It is known that structural changes to metal implant surfaces which increase roughness, for example by acid treatment, sandblasting or sintering powdered metal, can increase the *de novo* formation of bone, as well as enhance integration into existing bone.

25 In recent years it has become apparent that precise control of the topography of the surfaces of implants is preferred for both the regulation of cell-substrate response and to minimise topographical variations between implants [Mendonça et al (2009), Anselme and Bigerelle (2005)]. It would therefore be advantageous to find an efficient way of introducing precisely engineered nanotopographies onto bio-compatible substrates,  
30 preferably over a large area.

Desirable cell response to surfaces of implants includes enhanced osteoinduction and osteogenesis. Osteoinduction is the induction of stem cells to differentiate into mature bone cells. Osteogenesis is the formation of new bone by bone cells. It is known that in  
5 orthopaedic implant procedures, promotion of both of these factors offers advantages in terms of improved integration and performance of orthopaedic implants.

One of the most common materials used for orthopaedic applications is titanium, due to its light weight, high strength (and other mechanical properties) and excellent  
10 biocompatibility induced by its natural surface oxide ( $\text{TiO}_2$  or titania). The thickness of the oxide can readily be controlled through an anodic process and leads to a visible colour change of the material. It is considered that the surface chemistry does not change, only the thickness of the oxide, and so this is often used to provide a  
15 biocompatible colour coding for different devices, as overall oxide thickness has little impact on the cell/substrate response. It is considered that this is the case because cells have been shown by Lynch et al (2007) to only interact with the immediate surface of an implant.

It is possible to form  $\text{TiO}_2$  which is patterned at a nanoscale level (nanopatterned) by  
20 fabrication methods such as through-mask anodisation with aluminium oxide or block co-polymers. However, these approaches do not inherently offer free control of surface topography in terms of pattern design or allow the provision of multiple designs on one implant. Additionally, such known methods are not facilely applicable to curved substrates, which is an important consideration in view of the desired application in  
25 orthopaedic implants.

### **SUMMARY OF THE INVENTION**

The present inventors have realised that there is significant potential for improving the  
30 osteoinduction of medical implant interfaces through systematic manipulation of precisely

controlled nanotopographies. The inventors consider that the fabrication process disclosed herein provides an efficient, scalable and relatively easy route to the fabrication of bio-compatible, functional coatings on non-planar surfaces of substrates.

- 5 Previously, inventors from the same research group have shown manipulation of osteoinductive properties by careful control of the degree of misalignment of nanoscale features from a notional perfect lattice on polymeric substrates, as disclosed in WO 2007/057693. However, the substrate materials used in WO 2007/057693 are not suitable for load bearing applications as medical implants, in particular orthopaedic  
10 applications.

As mentioned above, a particularly suitable material for orthopaedic implants is titanium. Nanoscale features formed of nanopatterned  $\text{TiO}_2$  are known. For example, work by Ganesan et al. (2012), working not on Ti substrates but on Si substrates, discloses use  
15 of a rigid template or mould, pressed into a deformable material or liquid resist on a planar Si wafer to form a pattern. The resist is then cured in situ with UV, and the template then removed to leave a patterned resist on the Si wafer. This is then heated to burn off organic components to leave a  $\text{TiO}_2$  pattern.

- 20 Work by Richmond et al. (2011) similarly discloses formation of  $\text{TiO}_2$  nanopatterns onto a planar Si wafer. A sol-gel  $\text{TiO}$ -based resist is applied to the Si wafer by spin coating and then a soft PDMS mould is pressed against the resist. On heating, the resist cures and the mould is peeled away to leave the patterned cured resist.

- 25 Neither Ganesan et al. (2012) nor Richmond et al. (2011) are concerned with the formation of nanopatterns on a material suitable for use as a medical implant, nor are they concerned with the formation of nanopatterns on curved surfaces. There is no explanation in these disclosures of why or how corresponding nanopatterns would be formed on a non-planar (i.e. curved) substrate surface. This is a significant problem

given the fact that orthopaedic implants mainly present non-planar surfaces, and can exhibit a wide range of curvatures.

The present invention has been devised in order to address at least one of the above  
5 problems. Preferably, the present invention reduces, ameliorates, avoids or overcomes at least one of the above problems.

Accordingly, in a first preferred aspect, the present invention provides a method of forming nanostructures on a curved surface of a substrate, the method including:

10        providing a conformable template having template nanostructures,  
         providing a sol-gel comprising a patterning precursor,  
         applying the conformable template to the curved surface with the sol-gel  
interposed between the conformable template and the curved substrate to form a  
patterned coating of patterning precursor on the curved substrate, and  
15        subsequently carrying out thermal treatment of the patterned coating in order to  
develop the nanostructures.

In a second preferred aspect, the present invention provides a substrate with a curved  
surface with a patterned array of nanostructures, obtained by or obtainable by the  
20        method of the first aspect.

In a third preferred aspect, the present invention provides a medical implant with at least  
one curved surface, the curved surface having a patterned array of metal oxide  
nanostructures formed on it.

25        In a fourth preferred aspect, the present invention provides a medical implant according  
to the second aspect or the third aspect, for use in a method for treatment of the human  
or animal body by surgery or therapy.

In a fifth preferred aspect, the present invention provides a medical implant according to the second aspect or the third aspect, for use in bone implant surgery. For example, the bone implant surgery may include hip, knee or maxillofacial repair.

- 5 In a sixth preferred aspect, the present invention provides a use of a medical implant according to the second aspect or the third aspect in orthopaedic surgery.

The first, second, third, fourth, fifth and/or sixth aspects of the invention may be combined with each other, in any combination.

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Optional features of the invention will now be set out. These can be combined singly or in any combination with any aspect of the invention, unless the context demands otherwise.

- 15 Preferably, the conformable template has suitable flexibility to permit conformability to the curved surface of the substrate at relatively low pressure, in order to avoid unwanted deformation of the pattern to be produced on the substrate. For example, the Young's modulus of the material of the conformable template is preferably not greater than 30 MPa. For example, "soft" PDMS has Young's modulus of about 2 MPa. UV-treated
- 20 PDMS (UV-PDMS) has Young's modulus of about 7.7 MPa. HPDMS has Young's modulus of about 12 MPa. In contrast, X-PDMS has Young's modulus of about 80 MPa, but is brittle so is not considered to be suitable for conformal applications. Values for Young's modulus of these materials are taken from Schmitt et al (2012).

- The template nanostructures are preferably formed using a master substrate. The
- 25 master substrate may be formed as discussed below. The conformable template may be formed in contact with the master substrate, e.g. by casting the material of the conformable template against the master substrate, by embossing or by any other suitable approach.

One particularly suitable material for the conformable template is polydimethylsiloxane (PDMS). This can be cast against a master substrate. PDMS is considered to be particularly suitable for a number of reasons, including its ease of casting, its mechanic properties (permitting flexibility and conformability), and its permeability to organic solvents and its permeability to gases.

To achieve a suitable level of topographical control in terms of lateral dimensions and geometrical arrangement to form the master substrate, it is preferable to use electron beam lithography (EBL) to define an etch mask for use in fabricating the master substrate. EBL facilitates nanometre-level precision of control of the diameter (or other dimension), pitch and order of the nanofeatures to be formed in the master substrate.

A suitable process for forming the nanofeatures in the master substrate, based on the etch mask, is reactive ion etching (RIE). RIE can control etch depth to nanometre accuracy during the master substrate fabrication.

The term 'sol-gel' is used to propose a sol which can form a gel or is at least part gel. In this way, the sol-gel may be flowable. This is preferred particularly during the step of forming the patterned coating on the curved surface. Preferably, the patterning precursor comprises at least one material which is, or which is transformable to (e.g. by thermal treatment) metal oxide, such as titanium oxide. Zirconium oxide and aluminium oxide are also contemplated.

The patterning precursor may comprise a metal-based alkoxide. Metal-based alkoxides cross-link in the presence of water. This reaction may be chemically stabilised and the viscosity controlled by incorporating an organic solvent. Preferably, the organic solvent is selected to have a relatively low vapour pressure. This is preferred in order to increase the handling time of the sol-gel, which in turn allows for reliable and reproducible patterning of the sol-gel using the conformable template. The solvent is also able to escape the film during a subsequent curing stage for successful nanopattern

transfer, facilitated by the permeability of the conformable template to organic solvents and gases.

Thus, preferably, the sol-gel includes a metal-based alkoxide, water and an organic  
5 solvent.

It is considered that the solvent should have a vapour pressure below 50 mmHg (@ 20°C), more preferably not more than 40 mmHg (@ 20°C), more preferably not more than 30 mmHg (@ 20°C), more preferably not more than 20 mmHg (@ 20°C), more  
10 preferably not more than 10 mmHg (@ 20°C), preferably not more than 8 mmHg (@ 20°C), preferably not more than 6.5 mmHg (@ 20°C), preferably not more than 4 mmHg (@ 20°C), more preferably at or below 2 mmHg (@ 20°C). Suitable solvents include 2-methoxyethanol (6.17 mmHg (@20°C)), 1-hexanol (1.0 mmHg (@20°C)), 2-(2-Butoxyethoxy)ethyl acetate (BEEA) (0.0098 mmHg (@20°C)). A mixture of such  
15 solvents may be used to optimize processing. Of particular interest, for example, is a mixture of 1-hexanol and BEEA. At 50:50 mixture of 1-hexanol and BEEA has a vapour pressure of (0.50 mmHg (@20°C)). It was found that BEEA by itself was not suitable because BEEA is not miscible with water. However, BEEA is miscible in 1-hexanol. Additional solvents considered for application in the sol-gel, optionally in combination  
20 with one or more other solvents, and their vapour pressures, are:

- aniline (0.86 mmHg (@20°C))
- toluene (22 mmHg (@20°C))
- benzene (74 mmHg (@20°C))
- chloroform (91 mmHg (@20°C))
- 25 cyclohexane (85 mmHg (@20°C))
- hexane (119 mmHg (@20°C))
- methanol (93 mmHg (@20°C))

Preferably, the sol-gel is coated onto at least one of the conformable template or onto the  
30 substrate. One possible coating process is spin coating. This is particularly suitable for



coating the sol-gel onto the conformable template. In this case, the conformable template may be held flat during the spin coating process. Other coating processes may be used. For example the sol-gel may be sprayed onto the conformable template or onto the curved surface of the substrate. Spray coating is a method of particular use where  
5 the substrate has a complex shape. The substrate surface may, for example, have curvature in two orthogonal directions.

The sol-gel is therefore interposed between the conformable template and the curved surface of the substrate. The conformable template and the substrate are pressed  
10 together using a low but non-zero pressure. For example, preferably the pressure applied between the conformable template and the substrate is not more than 2 bar. Preferably, this pressure is not more than 1 bar. This step results in formation of a patterned coating of sol-gel between the curved surface of the substrate and the structured surface of the conformable template, through either a printing-type, an  
15 imprinting-type or an embossing-type process.

Preferably, the conformable template, sol-gel and substrate are held together and the sol-gel is gelled (cured). The assembly of conformable template, sol-gel and substrate may be held together via one or more releasable fixings.

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In some embodiments, it can be useful to include methanol in the solvent mix, for example with 1-hexanol. This is despite methanol having a relatively high vapour pressure of 93 mmHg @20°C. The advantage of including methanol is found particularly when the sol-gel is applied to the conformable template, rather than when the sol-gel is  
25 applied directly to the substrate. The inclusion of methanol allows the sol-gel to be diluted sufficiently so that the thickness of the coating can be formed to be small enough to be below a thickness threshold to allow crack-free annealing, and yet provides adequate gelification during the curing stage to retain nanofeatures. This is an advantage compared with where the solvent mix comprises only low vapour pressure  
30 solvents. In a preferred embodiment, the solvent mix comprises about 1 part methanol to

about 1 part 1-hexanol. The resultant vapour pressure of the solvent mix is then about 46 mmHg @20°C. Thus, a curved substrate can be treated by forming a layer of the sol-gel onto the conformable template by spin coating, and then performing contact printing to transfer the coating to the curved substrate.

5

Curing may be promoted using heat and/or radiation. Where curing is promoted using heat, preferably the temperature is at least 90°C, more preferably at least 100°C. In the case of a 200nm thick coating, for example, the curing process requires at least 8 minutes, more preferably at least 10 minutes. The preferred permeability of the material of the conformable template to organic solvents and gases allows these components to escape during the curing stage. This permits faithful reproduction of the shape of the nanostructures formed on the conformable template.

10

After curing, the conformable template is preferably removed from the substrate. The patterned sol-gel coating preferably adheres preferentially to the surface of the substrate. As will be understood, the sol-gel incorporates the patterning precursor in the form of the metal-based alkoxide, for example.

15

The substrate having the patterned coating is then subjected to a thermal treatment. Suitable thermal treatments depend on the nature of the patterning precursor and the desired material of the nanostructures. In the case of metal oxides, preferably the thermal treatment is suitable to achieve at least partial sintering of the metal oxide particles. There may be provided an interim thermal treatment stage, before the sintering stage, to allow burn-off of organics from the patterned coating.

20

In the case of titania, the sintering temperature is significant because different phases of TiO<sub>2</sub> are observed to result from different sintering temperatures. Sintering at about 500°C produces a coating predominantly of the anatase phase, whereas sintering at around 700°C produces a coating predominantly of the rutile phase. Sintering at lower temperatures, for example at around 300°C produces an amorphous coating. Preferably therefore, at least in the case of titania, the sintering temperature is at least 450°C. For

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orthopaedic applications, anatase may be the preferred phase, and therefore the sintering temperature may be up to 550°C, for example. However, sintering at higher temperatures, e.g. up to about 800°C may be suitable for some applications.

- 5 Preferably, the ramp rate of temperature increase up to the sintering temperature is relatively low, for example not greater than 5°C/min, more preferably not greater than 3°C/min. For example, a ramp rate of about 2°C/min is found to be suitable. Using ramp rates of this nature, the residual level of carbon in the coatings can be reduced to a suitable level. Additionally, this approach assists in the control of the thermal stress
- 10 experienced by the patterned coating. It is found that such thermal treatments can provide suitable integration of the particles in the coating with a suitably low residual carbon content (less than 10%, more preferably about 5.5%, which is comparable to the residual carbon content found on control surfaces).
- 15 Similar thermal treatments can be used for different compositions.

- Preferably, the substrate comprises a bio-compatible material. Suitable bio-compatible materials are known. In the case of orthopaedic implant devices such as used in joint repair or joint replacement surgery, the material of the substrate requires significant
- 20 mechanical performance. Preferably, therefore, the substrate is formed of at least 50% by mass of a material with Young's modulus of at least 50 GPa. Additionally or alternatively, the substrate is formed of at least 50% by mass of a material with ultimate tensile strength (UTS) of at least 100 MPa, more preferably at least 200 MPa.
- 25 Additionally or alternatively, the substrate is formed of at least 50% by mass of a material with fracture toughness  $K_{Ic}$  of at least 10 MPa.m<sup>-0.5</sup>. The substrate is preferably metallic. However, as will be understood, the substrate may be polymeric, since there are suitable polymers available with satisfactory mechanical performance and biocompatibility.

- The pattern in which the nanostructures are arranged, and also the shape and size of the
- 30 individual nanofeatures, can be selected from a wide range of possible options. In some

preferred embodiments of the invention, it is preferred that the nanostructures promote osteoinductive effects. For this reason, the preferred patterns comprise features in a near-square (NSQ) arrangement, preferably with features randomly displaced. This is explained more generally and in more detail below.

5

The nanofeatures preferably comprise either pillars or pits, and most preferably comprise pillars, as these are seen to provide a higher level of osteoinductive stimulant than equivalent pits. Comparisons of pillar topography with planar counterparts indicate that osteogenic biochemical pathways are exclusively activated on pillar surfaces, as are  
10 mechanotransduction pathways involving integrin signalling.

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Preferably, the nanostructures on the curved surface of the substrate are arranged in a pattern based on a notional symmetrical lattice in which the distance between nearest neighbour notional lattice points is  $C$ . Typically,  $C$  is between 10 nm and 10  $\mu\text{m}$ . The nanostructures are locally mis-ordered such that the centre of each topographical feature is a distance of up to one half of  $C$  from its respective notional lattice point. Such mis-order provides beneficial effects related to biocompatibility and cell response.

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Preferably, the nanostructures are protrusions from and/or recesses into the surface of the substrate. In particular, the topographical features may include upstanding pillars. Additionally or alternatively, the topographical features may include pits.

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Preferably,  $C$  is at least 20 nm, at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, at least 120 nm, at least 130 nm, at least 140 nm, at least 150 nm, at least 160 nm, at least 170 nm, at least 180 nm, at least 190 nm, at least 200 nm, at least 210 nm, at least 220 nm, at least 230 nm, at least 240 nm, at least 250 nm, at least 260 nm, at least 270 nm, at least 280 nm, at least 290 nm or about 300 nm.

Preferably, C is at most 9  $\mu\text{m}$ , at most 8  $\mu\text{m}$ , at most 7  $\mu\text{m}$ , at most 6  $\mu\text{m}$ , at most 5  $\mu\text{m}$ , at most 4  $\mu\text{m}$ , at most 3  $\mu\text{m}$ , at most 2  $\mu\text{m}$ , at most 1  $\mu\text{m}$ , at most 900 nm, at most 800 nm, at most 700 nm, at most 600 nm, at most 500 nm, at most 400 nm.

- 5 The most preferred range for C is between 30 nm and 3  $\mu\text{m}$ .

Preferably, the height or depth (e.g. the average height or depth) of the nanostructures is at least 5%, more preferably at least 10%, of C from the remainder of the local surface of the substrate. For example, the height or depth of the nanostructures may be at least

- 10 10 nm.

Preferably, each nanostructure has the same shape. The nanostructures may be cylindrical pits or pillars, cuboid pits or pillars, hemi-spherical pits or pillars, part-spherical pits or pillars, or another regular shape.

- 15

Preferably, the diameter of the nanostructures is at least 10%, more preferably at least 20%, at least 30%, at least 40% or at least 50%, of C. For example, the diameter of the topographical features may be at least 20 nm.

- 20 Preferably, the centre of each nanostructure is at most 45%, more preferably at most 40%, at most 35%, at most one third, at most 30%, at most 25%, at most 20%, at most 15%, at most 10% or at most 5%, of C from its respective notional lattice point.

- 25 Preferably, for at least 50% of the nanostructures, the centre of each nanostructure is between one tenth and one quarter of C from its respective notional lattice point. More preferably, at least 60%, at least 70%, at least 80% or at least 90% of the nanostructures satisfy this criterion. The lower limit for the distance of the centre of each nanostructure from its respective notional lattice point is preferably at least 12 % of C, at least 14% of C or at least 16% of C. The upper limit for the distance of the centre of each nanostructure

from its respective notional lattice point is preferably at most 22% of C, at most 20% of C or at least 18% of C.

The nature of the symmetry on which the notional lattice is based may be selected from a  
5 parallelogram lattice, a rectangular lattice, a square lattice, a rhombic lattice, a trigonal lattice and a hexagonal lattice. Preferably, the notional lattice is either a rectangular lattice or a square lattice.

In the method of manufacturing the nanostructures, the design of the conformable  
10 template is preferably carried out. In turn, this requires designing a master substrate, where a master substrate is used to form the conformable template. Thus, preferably the method includes the steps of designing the notional symmetrical lattice, applying a degree of mis-order to the notional symmetrical lattice by requiring that the centre of each nanostructure, or feature corresponding to each nanostructure, is up to one half of  
15 C from its respective notional lattice point, thereby designing a mis-ordered lattice, and manufacturing the master substrate according to the mis-ordered lattice.

Preferably, the degree of mis-order is applied to each notional lattice point by a calculation step in which a random number is generated and used to provide one or more  
20 displacement amounts to said notional lattice point. For example, for each lattice point of a rectangular or square lattice, a random displacement along one axis may be applied, followed by a random displacement along an orthogonal axis. For a non-orthogonal lattice (e.g. a parallelogram lattice, hexagonal lattice or trigonal lattice), these random displacements may be made along axes of the lattice, or along orthogonal axes.  
25 Typically, the random number generated is operated on using a multiplier, that multiplier corresponding to the fraction of C corresponding to the desired maximum mis-order of the array of topographical features.

Preferably, the method comprises the step of forming an array of topographical features  
30 using electron beam lithography. This array may be formed on the surface of the master

substrate. The master substrate need not itself be a biocompatible substrate suitable for implantation into the human or animal body.

The master substrate is then preferably used to create the conformable template.

- 5 Typically, the conformable template provides the “negative” topographical features to those of the master substrate. The conformable template is then used as set out above, to form a patterned coating of patterning precursor on the curved substrate.

- 10 The diameter of the nanofeatures (e.g. in the case of pillars) is preferably between 40 and 100nm, although larger diameters may also be suitable. The height of the nanofeatures (e.g. in the case of pillars) is preferably between 8 and 80nm. The height of the nanofeatures may be at least 15nm for example. The height of the nanofeatures may be at most 40nm for example.

- 15 Further optional features of the invention are set out below.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

- 20 Embodiments of the invention will now be described by way of example with reference to the accompanying drawings in which:

Fig. 1 shows a schematic plan view of a nanotopography for use with an embodiment of the invention.

Fig. 2 shows a schematic plan view of a nanotopography for use with an embodiment of the invention.

- 25 Fig. 3 shows a schematic plan view of a nanotopography for use with an embodiment of the invention.

Figs. 4-6 show SEM micrographs of different nanotopographies.

Fig. 7 shows SEM images and FFT images of the different nanotopographies of Figs. 4-6.

Figs. 8A-E show steps in the manufacture of nanopillars on a substrate surface.

Figs. 9F-9H show surface characterisation of a curved substrate treated according to an embodiment of the invention.

Figs. 10I-K show TEM analysis of a surface treated according to an embodiment of the invention.

5 Fig. 11A shows a low magnification SEM image of a curved surface of a Ti rod with a nanopatterned region indicated by an arrow. Fig. 11B shows a higher magnification SEM image of the nanopillars formed on the curved surface of the Ti rod.

Fig. 12 shows the result of Raman analysis of TiO<sub>2</sub> patterns subjected to different heat treatments.

10 Fig. 13 shows a bar graph summarising the average amount of protein (in pixel area) produced per cell for each sample, for different surface treatments.

#### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS, AND FURTHER OPTIONAL FEATURES OF THE INVENTION**

15

A suitable process for forming the master substrate for use in an embodiment of the invention will first be described. The master substrate is then used to form the conformable template, which in turn is used to form the nanostructures on a curved surface of a substrate.

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A suitable pattern having a desired degree of mis-order is produced in a master. This master is formed of silicon in this embodiment, since patterning of silicon is well-understood. The silicon master is near atomically flat before patterning and is sufficiently conducting during electron exposure in e-beam lithography to avoid sample charging.

25

The desired pattern is generated by a computer program in which a suitable notional lattice is defined and each topographic feature is randomly displaced along the axes of the lattice by a random value. The software generates a file suitable for an electron beam lithography tool to read and execute. The silicon substrate is coated with a polymeric material, generally termed resist, which is susceptible to electron exposure. In

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the regions where the electron beam lithography tool exposes the resist, the regions will



either be removed or left behind after development. This is determined by the type of resist used, generally termed positive or negative resist. Such considerations as the nature of the resist and the nature of the substrate will be well understood by a person skilled in the art.

5

Known suitable electron beam lithography tools have a grid resolution of 5 nm. Recently, more advanced electron beam lithography tools have become available that have a grid resolution of 1 nm. Suitable electron beam lithography tools will be known to persons skilled in the art. The resolution of the position of the topographic features is determined by the grid resolution of the electron beam lithography tool. However, there is also a stochastic displacement as a result of signal noise, temperature variations etc.

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After patterning of the resist on the surface of the silicon, the pattern formed in the resist can be transferred to the silicon through a reactive ion etch process, well known to the skilled person. This yields a silicon surface with a topographic pattern. The silicon surface can be used directly as the master substrate. Alternatively, a nickel shim can be formed from the silicon surface by electro plating, a process well-known and used in the optical storage industry (CDs and DVDs). To make a nickel shim the silicon is first coated with a thin conducting metal film which subsequently acts as an electrode during the galvanic electroplating. The formed nickel shim is a negative copy of the silicon surface and can be used as the subsequent master substrate.

15

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Fig. 1 shows a schematic plan view of a nanotopography 100 formed from nanopits or nanopillars 104, based on a notional square lattice (the notional lattice points being defined by the intersections of straight dashed lines). The distance between nearest neighbour notional lattice points is  $C$ . As shown in exaggerated form in this drawing, the centres of the nanopits 104 are offset from their respective notional lattice points. The maximum offset is shown in this case as defined by a dashed circle 102 or radius about one third of  $C$  surrounding each notional lattice point. However, it should be noted that

25

the maximum offset need not be defined by a circle, but could be defined by a square (or rectangle) centred on each notional lattice point.

Fig. 2 shows a schematic plan view of a different nanotopography 200 formed from  
5   nanopits or nanopillars 204, based on a notional rectangular lattice (the notional lattice points being defined by the intersections of straight dashed lines).

Fig. 3 shows a schematic plan view of a different nanotopography 300 formed from  
nanopits or nanopillars 304, based on a notional hexagonal lattice.

10

In each of the schematic embodiments, the mis-order is apparent, but the arrangement of nanotopographical features is not truly random, due to the (statistically) relatively narrow distribution of distances between nearest neighbour topographical features. In other words, the arrangement of the topographical features does not allow for the creation of  
15   large gaps between features on the surface.

Fig. 4 shows an SEM micrograph of an orthogonal array of 120 nm diameter pits formed in single crystal silicon, the pits being 100 nm deep with 300 nm centre to centre spacing.

20   Fig. 5 shows an SEM micrograph of an orthogonal array of 120 nm diameter pits formed in single crystal silicon, the pits being 100 nm deep with 300 nm centre to centre spacing. Each pit has been randomly displaced from its respective notional lattice point by +/-20 nm.

25   Fig. 6 shows an SEM micrograph of an orthogonal array of 120 nm diameter pits formed in single crystal silicon, the pits being 100nm deep with 300 nm centre to centre spacing. Each pit has been randomly displaced from its respective notional lattice point by +/-50 nm.

Fig. 7 (bottom row) shows SEM micrographs of the orthogonal and nearly orthogonal nano pit arrays of Figs. 4-6. The corresponding FFT images (top row) illustrate the decrease in long range order for the more disordered nanotopographies.

- 5 Where relevant, in the embodiments described herein, the degree of mis-order applied to a notional lattice uses the notation  $\pm X$ , denoting that the maximum allowed deviation of each feature from its notional lattice point is a distance  $X$  along one axis of the lattice and a distance  $X$  along another axis of the lattice. For each feature, therefore, a deviation of between (and including) 0 and  $X$  is allowed, along each axis. Note that it is also possible
- 10 to specify that the degree of mis-order along one axis is different to the degree of mis-order along another axis. Such asymmetrical mis-order would be denoted  $\pm X_a$  and  $\pm X_b$ , indicating the mis-order applied along axis  $a$  and the mis-order applied along axis  $b$  of the notional lattice.
- 15 Surface design at the nanoscale has been a popular route to induce osteogenesis with musculoskeletal applications in mind. However, to date, there has been limited success in realising this in vivo. This ambition is driven, in part, by emerging concerns related to the use of growth factors such as BMP 2 [Subach et al (2001), Garrison et al (2007), Carragee et al (2011)]. Researchers from the inventors' group have previously detailed
- 20 the ability of disordered nanotopography (NSQ – near square) to modulate skeletal stem cells, more commonly referred to as mesenchymal stem cells, along the osteogenic lineage [Dalby et al (2007)]. These nanopatterns consist of 120 nm diameter pits arranged with an average pitch of 300 nm and with a random offset of up to 50 nm from a perfect square grid. We hypothesised that NSQ nanopits could be harnessed to drive
- 25 and switch immunoselected skeletal osteoprogenitors to a directed osteogenic phenotype in vivo. The same nanotopography was also able to differentiate human embryonic stem cells (HUES-7) along the mesoderm lineage with markers of skeletal stem cells (STRO-1, CD44, CD63, ALCAM) [Kingham et al (2013)]. In contrast, a highly ordered nanotopography (SQ) was observed to be capable of promoting skeletal stem
- 30 cell self-renewal, by enhancing symmetric cell division in the absence of soluble,

chemical differentiation-inducing factors [McMurray et al (2011)]. However, these previously reported findings were carried out using polymeric substrates (polycarbonate and polystyrene) through the route of hot embossing or injection moulding and so, until now, these highly osteogenic and reproducible nanopatterns have not found translational use in orthopaedics. A particular challenge is thus to provide these results in a relevant, load bearing material for musculoskeletal applications [Greer et al (2007)].

One of the most commonly used materials for orthopaedic applications is titanium given its light weight, high strength and excellent biocompatibility induced by its natural surface oxide (titanium dioxide or titania) [Buechel and Pappas (2011)]. The thickness of the oxide can readily be controlled through an anodic process and leads to a visible colour change of the material [Van Gils et al (2004)]. As the surface chemistry does not change with the thickness of the oxide, this is often used to provide a colour coding for different devices. Our preferred aim is to utilise this and produce our osteogenic nanotopography in a TiO<sub>2</sub> based surface coating. The formation of nanopatterned titania can be made by nanofabrication methods such as through-mask anodisation using aluminium oxide or block co-polymer masks [McNamara et al (2011), Sjöström et al (2009) and (2013)]. Although facile fabrication approaches exist, these do not offer the precise control of surface topography required to generate NSQ nanotopographies. Thus we have used an organic-titanium sol-gel precursor to form the patterned titania on an implant with the required topographical control. Such precursors have previously been demonstrated to have the ability to be nanopatterned using a soft, conformable master and then sintered to form titania [Richmond et al (2011), Lim et al (2010), Yoon et al (2009), Ganesan et al (2012)]. Here we report on the combination of the technology with our prior knowledge of osteogenic nanopatterns to enable a simple method of fabricating biocompatible, functional coatings on titanium surfaces.

In the preferred embodiments of the invention, the nanofeatures produced on the curved surface of the substrate are based on a square (orthogonal) notional lattice, with mis-order as described above. This is referred to here as a near-square arrangement (NSQ).

According to a preferred embodiment of the invention, the conformable template is a template made by casting polydimethylsiloxane (PDMS) against a patterned silicon master substrate.

5

The template nanostructures selected in the preferred embodiment is a near-square (NSQ) array of nanopillars. The nanopillars are randomly displaced up to 50nm from a 300nm pitch square notional lattice. To achieve the required level of topographical control in terms of lateral dimensions and geometrical arrangement, electron beam

10 lithography (EBL) is used to fabricate the master substrate, by defining the etch mask in a bi-layer of polymethylmethacrylate (PMMA) coated Si. To produce a mould for PDMS pillars, the positive-tone PMMA may itself be utilised as the etch mask. Conventional RIE then proceeds. Thereafter a trichloro silane mono-layer is deposited onto the master substrate to facilitate compliant demoulding of PDMS post-casting. Sylgard 184 from  
15 Dow Corning is cast upon the moulds in a 1:10 / curing agent:monomer weight ratio, and, once cast, it is cured in an oven at 70°C overnight. Once cured the PDMS templates are peeled from the master mould and trimmed using a scalpel blade.

In the preferred embodiment, the sol-gel is prepared by mixing 0.96 ml of diethanolamine  
20 (99%) with 5.54 ml of 1-hexanol (99%) and 0.10 ml of deionised water. The mixture is vigorously stirred for 10 minutes before adding 3.40 ml of Ti(OBu)<sub>4</sub> (97%) while stirring. Stirring continues in a sealed vial for 2 hours to ensure complete dissolution. The shelf life of this sol-gel precursor is in excess of 14 months when stored at room temperature.

25 The sol-gel is spin-coated onto the conformable template (for example, at 9 krpm for 7 seconds). In alternative embodiments, the sol-gel is applied to the curved surface of the substrate, for example by spraying. Spraying or other application techniques are of particular interest when the substrate has a complex shape.

The patterned PDMS conformable template is then pressed against the curved surface of the substrate with the sol-gel interposed between the conformable template and the curved substrate.

- 5 In a preliminary investigation, the patterned PDMS conformable template, with the sol-gel spin-coated onto the patterned surface, is pressed against a planar surface of a substrate. In this way, the sol-gel is interposed between the conformable template and the substrate. The pressure applied is the weight of the PDMS conformable template, corresponding to a pressure of 44 Pa. The conformable template and the substrate  
10 surface therefore control the shape of the sol-gel in the subsequent processing. This assembly is then heated at 120°C for 10 minutes before removing the conformable template and sintering the sample at 500°C with a ramp rate of 2°C/min, to form TiO<sub>2</sub> nanostructures on the substrate surface. The TiO<sub>2</sub> nanostructures are predominantly in the anatase phase.

15

- In an embodiment of the invention, TiO<sub>2</sub> nanostructures were formed on a curved surface of a Ti substrate. In this case, the Ti substrate was a Ti rod of diameter 10mm. By capitalising on the prolonged handling time of the novel sol-gel chemistry disclosed herein, the imprinting process may accommodate multiple iterations of contact-print  
20 lithography upon non-planar surfaces. Thus a large area may be patterned through serial contact-printing. This can be achieved by spin coating the sol-gel onto the template and exploiting the conformability of the PDMS template to print nanopatterned sol-gel layers onto non-planar, large areas of titanium (for example).

- 25 To achieve the required level of topographical control in terms of lateral dimensions and geometrical arrangement, we used electron beam lithography (EBL) to fabricate the master substrates. EBL facilitates nanometre precise control of the diameter, pitch and order of the nanofeatures during the master fabrication [Hartley et al]. Similarly, reactive ion etching can control the etch depth to nanometre accuracy during the pattern transfer  
30 to the substrate [Hsu et al (2008)]. Initial investigations during the translation from

polymer to titania based substrates indicated a need for a range of different master substrates in order to evaluate the optimal pattern geometry for skeletal stem cell differentiation on the titania substrates. A library of master substrates was manufactured with different feature dimensions and polarity. The flexible template was made by casting PDMS against the master substrate which facilitated patterning of non-planar surfaces (such as hips, screws and tools) to be susceptible for this precision patterning. Pillar diameters were varied from 40 to 100 nm and the height from 8 to 80 nm following heat treatment (sintering). The fabrication process is summarised in Figs. 8A-E. Figs. 9F-H (there is no Fig. 9A-E) and Figs. 10I-K (there is no Fig. 10 A-H) show results from surface characterization testing of the substrate.

Figs. 8A-E show an example process flow for surface nanostructuring. Step A: PDMS template 12 is made from a silicon master 10. Step B: a sol-gel layer 16 is coated onto a Ti substrate 14 and imprinted with the PDMS template 12. Step C: the “sandwich” of sol-gel layer 16 between the substrate 14 and the PDMS template 12 is cured. Step D: the template 12 is released from the substrate 14. Step E: the imprinted substrate 14 is annealed to form nanopatterned  $\text{TiO}_2$ .

Figs. 9F-H show results of surface characterisation. Fig. 9F shows a plan view SEM image of nanopillars. Fig. 9G shows an AFM based 3D perspective of the nanopillars. Fig. 9H shows an image of a rod substrate with its curved surface nanopatterned at three separate regions (regions highlighted by markers).

Fig. 10I shows a HAADF survey image of a cross-section from a sintered sol-gel coated piece of cpTi (commercially pure titanium). Fig. 10J shows a plot of internal ELNES spectra from different regions of the subsection area. Fig. 10K shows a close-up subsection image and series of phase maps for the subsection site, each map being labelled appropriately. For the single colour images, the brighter a pixel, the stronger the fit to the appropriate spectrum displayed in the top right. The ‘Composite’ image of Fig. 10K is colour mapped rather than brightness mapped and the colours correspond to the

ELNES key. For all of these images the scale bar is the same as the 'Ti metal' plot, 20 nm.

Fig. 11A shows an SEM overview image of the Ti rod circumference post sinter with a  
5 superimposed arrow to indicate the boundary of the nanopattern, scale bar = 1 mm. Fig.  
11B shows an SEM close-up image of the nanopattern, scale bar = 1  $\mu$ m.

Metal-based alkoxide cross-links in the presence of water. This reaction may be chemically stabilised and the viscosity controlled by incorporating an organic solvent.

10 The sol-gel synthesised in this work featured titanium-butoxide mixed with water, diethanolamine (stabilising agent) and 1-hexanol.

In more detail, the solvent contains alcohol in order to facilitate sol-gel alkoxide formation. As explained previously, the alcohol should preferably have a relatively low vapour  
15 pressure. 2-methoxyethanol with vapour pressure 6.17mmHg (20°C) was tested and could be used if spun on the substrate for 1 second @ 6k rpm and imprinted immediately (sub-3 seconds). 1-hexanol as the solvent is preferred due to possessing a vapour pressure of 0.99mmHg (20°C) enabling a longer spin coat (7 seconds) and extended (sub-30 seconds) handling time. Even lower vapour pressures extend the handling time  
20 further. A mixture of 50%parts 1-hexanol and 50%parts 2-(2-Butoxyethoxy)ethyl acetate (BEEA) was found to provide a resultant vapour pressure of 0.50mmHg so to increase the handling time 8 fold compared with 100% 1-Hexanol solvent. The benefits of the lower vapour pressure solvent are: spin duration (or more generally, coating time) and handling time are increased to allow more time to align imprint stamps or manipulate the  
25 device before the coating dries, reduced coating thickness which reduces thermal stress across the coating, thinner edge-beading on devices thus greater homogeneity of coating thickness, varying coating duration will produce a spectrum of post-annealed colours useful for colour coding products.



Previous work by Yoon et al (2009) and Richmond et al (2011) used solvents with relatively high vapour pressures (6 – 45 mmHg (20 °C)) leading to rapid evaporation of the solvent, reducing the handling time of the material during the patterning phase to a few seconds. As explained above, we identified a suitable solvent to provide an  
5 extended handling time allowing for reliable and reproducible patterning of the sol-gel using the nanopatterned PDMS TEMPLATE. The solvent needs to be able to escape the film during the curing stage for successful nanopattern transfer which is facilitated by the permeability of PDMS to organic solvents and gases [Abate et al (2008)]. To assess the chemical quality of the formed titania surface, we examined the surface composition  
10 using x-ray photoelectron spectroscopy (XPS). Following sintering above 500°C, the carbon content (5.5%) was observed to be comparable to the tested commercially pure titanium (cpTi) control (4.5%). The polymorph of the formed titania could be controlled by the annealing temperature: annealing at 300°C produced amorphous titania, 500°C produced anatase and 700°C formed rutile. This is beneficial to implant devices to not  
15 only ensure biocompatibility, but also to address specific crystal phases which are beneficial at the implant surface [He et al (2008), Sollazzo et al (2007)]. Fuller details on XPS and Raman analysis for phase analysis is provided below. For this work we used anatase films as this is the most commonly studied phase in the literature [Chen et al (2008)].

20

Raman analysis of the sol-gel-derived coating on cpTi was performed on a Renishaw InVia Raman microscope using a 785 nm wavelength laser. The Renishaw CCD sensor was utilised at 5 exposures per second and 10 traces were accumulated for each spectrum to improve signal-to-noise. The resultant spectra are displayed in Fig. 12. The  
25 sol-gel coating which was annealed at the lowest tested temperature (300°C) is dominated by noise as the coating is amorphous. The coating annealed at 500°C possesses all of the characteristic peaks of anatase (395, 517 and 638 cm<sup>-1</sup>) and the coating annealed at 700°C shows the expected rutile peaks (447 and 612 cm<sup>-1</sup>).

A SAGE 100 system (Specs GmbH, Germany) was used as for the XPS analysis. Base pressure in the analysis chamber was approximately  $2\text{e}^{-7}$  mbar. The X-ray source was MgK $\alpha$  operated at an anode voltage of 12.5 kV and 250 W of power. Spectra were recorded, following a 50 minute Ar sputter, at a take-off angle of 90 degrees. This was carried out to remove the natural oxide formed at the surface as to get more realistic elemental composition of the bulk material. The pass energy for the hemispherical analyser was 50 eV for the survey scans used to determine the elemental composition. Spectra were analysed using casaXPS software, and the elemental composition was determined by integration of peak areas using a standard Shirley background.

XPS was performed on a variety of titania precursor coatings processed with a range of annealing parameters to determine the preferential conditions for removing carbon from the coating. In all cases the furnace was held at maximum temperature for 30 minutes before allowing the furnace to cool down to room temperature overnight.

It was discovered that by reducing the annealing rate from 10°C/minute (as suggested in the literature [Richmond et al (2011)]) to 2°C/minute, the resulting carbon quantity may be significantly reduced from 16% to about 6% for the precursor using 2-methoxyethanol as the diluent. A slight reduction of less than 1 % was recorded when the precursor was adapted by replacing 2-methoxyethanol with 1-hexanol, however this sub 1 % change is negligible as the detection limit for the XPS machine used is about 1 %. On the same basis an increase in annealing maximum temperature from 500°C to 700°C may also be considered ineffective at reducing the XPS detectable carbon. A control sample of cpTi was solvent cleaned and subjected to the same XPS analysis as a control to determine the intrinsic level of carbon detectable in cpTi. The result was a carbon level of 4.5%. The results are tabulated in Table 1 below.

Table 1 - XPS analysis of sol-gel coated samples where various chemical and annealing parameters have been varied to reduce carbon content. The lowest level detected was 5.5% and the final column contains a control sample of bulk cpTi foil.

| <b>Surface:</b>                            | <b>Ti</b>                        | <b>Ti</b>                        | <b>Ti</b>                        | <b>Ti</b>                            | <b>Ti<br/>(control)</b> |
|--------------------------------------------|----------------------------------|----------------------------------|----------------------------------|--------------------------------------|-------------------------|
| <i>Coating:</i>                            | <i>TiO<sub>2</sub> precursor</i> | <i>TiO<sub>2</sub> precursor</i> | <i>TiO<sub>2</sub> precursor</i> | <i>TiO<sub>2</sub><br/>precursor</i> | <i>none</i>             |
| <i>Precursor<br/>solvent:</i>              | <i>2-<br/>methoxyethanol</i>     | <i>2-<br/>methoxyethanol</i>     | <i>1-hexanol</i>                 | <i>1-hexanol</i>                     | <i>N/A</i>              |
| <i>Anneal<br/>maximum<br/>temperature:</i> | <i>500 °C</i>                    | <i>500 °C</i>                    | <i>500 °C</i>                    | <i>700 °C</i>                        | <i>N/A</i>              |
| <i>Anneal rate:</i>                        | <i>10 °C /minute</i>             | <i>2 °C /minute</i>              | <i>2 °C /minute</i>              | <i>2 °C /minute</i>                  | <i>N/A</i>              |
| <i>Titanium level:</i>                     | <i>25.0%</i>                     | <i>28.4%</i>                     | <i>31.0%</i>                     | <i>30.8%</i>                         | <i>25.0%</i>            |
| <i>Oxygen level:</i>                       | <i>58.2%</i>                     | <i>65.3%</i>                     | <i>63.5%</i>                     | <i>62.6%</i>                         | <i>70.5%</i>            |
| <i>Carbon level:</i>                       | <i>16.8 %</i>                    | <i>6.3 %</i>                     | <i>5.5 %</i>                     | <i>6.6 %</i>                         | <i>4.5%</i>             |

The conclusion of the XPS analysis is that the preferential annealing conditions for the 1-hexanol based precursor was to anneal with a slow ramp rate of 2°C/minute to a

5 temperature of 500°C (retain the sample at this level for 30 minutes before allowing the furnace to cool overnight) in order to achieve a level of carbon comparable to that present in cpTi.

A natural key aspect of the titania films is a firm integration with the underlying titanium

10 substrate. Scanning transmission electron microscopy (TEM) and electron energy loss spectroscopy (EELS) analysis were carried out on a cpTi cross-section sample containing the sol-gel coating which had been sintered at 500°C to examine the integration between the coating and the substrate. Integration appears firm with no signs of delamination. Moreover, EELS also confirmed that the sol-gel layer was crystallised

15 into a slightly porous almost entirely anatase phase, the preferential phase for orthopaedic applications [Chen et al (2008)]. Below the level of the original cpTi surface, a dense layer of rutile-like crystals forms, with the interface to the metal containing a few

nm of amorphous titania – this rutile/anatase layer of combined thickness about 40 nm probably formed from oxidation of the metal during annealing.

Figs. 10I-K display an overview of the TEM analysis. A high-angle annular dark field

(HAADF) survey image is displayed in Fig. 10I which provides a high-resolution image of the interface. At the top of the cross-section image is the titanium substrate, at the bottom is the porous, sintered sol-gel layer. A subsection, as highlighted with the box in Fig. 10I, was used for the phase analysis. Several internal energy-loss near-edge structure (ELNES) spectral references for phase were selected based on known spectra defined in literature [Gloter et al (2009)]. The internal spectra are displayed in Fig. 10J. Spatial phase maps depicting the quality of each spectrum's fit to the subsection area are shown in Fig. 10K. In the four specific-phase phase maps the brightest pixels correspond to the strongest fit with the corresponding spectrum. In the final 'Composite' image, the map is colour coded by superimposing the different phases into one image.

In order to evaluate the most osteoinductive nanopillar dimensions in the present work, a range of heights and diameters of circular NSQ pillar were fabricated as described above. Each pattern covered a square area of 5x5 mm. The tested nanofeature dimensions are shown in Table 2.

Table 2 - Array of tested nanopillar dimensions, 'x' represents a tested dimension.

| <b>Diameter (nm) →</b> | <b>40</b> | <b>70</b> | <b>100</b> |
|------------------------|-----------|-----------|------------|
| <b>Height (nm) ↓</b>   |           |           |            |
| <b>8</b>               | x         | x         | x          |
| <b>15</b>              | x         |           | x          |
| <b>20</b>              |           |           | x          |
| <b>25</b>              | x         |           |            |
| <b>30</b>              |           | x         |            |

| <b>Diameter (nm) →</b> | <b>40</b> | <b>70</b> | <b>100</b> |
|------------------------|-----------|-----------|------------|
| <b>Height (nm) ↓</b>   |           |           |            |
| <b>40</b>              |           |           | x          |
| <b>60</b>              |           | x         | x          |
| <b>80</b>              | x         |           |            |

The surfaces were each cultured in one well of a six well plate and seeded with 3 ml of bone marrow cells at a concentration of 10,000 cells/ml. Bone marrow contains a large variety of components including MSCs, osteoprogenitors and osteoblasts. Thus analysis with this source of cells may not conclude whether the topographies tested upregulate the transformation from an MSC to an osteoblast but it is a very relevant source of cells because in in vivo trials individual cell phenotypes will not be found in exclusive populations. The cells were cultured upon the samples for three weeks. At the end of which the samples were fluorescently tagged to identify the cell nucleus (DAPI stain), cell cytoskeleton (actin stain) and bone related proteins osteocalcin (OCN) and osteopontin (OPN) (antibody stain). The production of OCN and OPN are known early indicators of bone formation. By quantifying the amount of OCN and OPN produced per surface one may make a reasonable assumption about the preferential pillar dimensions for bone formation. To analyse the surfaces, a 3 x 3 array of images at 2 mm pitch were captured using a 10x microscope objective which gave a square field of view with side 0.79 mm. The area of protein visible in each of the nine images per sample was measured with Cell Profiler software [Carpenter (2006)]. The parameters used were thresholds for intensity and size. Primarily the software identified which pixels were within the manually set intensity range – which corresponded to the fluorescent light emitted by the target protein – then disregarded any pixels which were not sufficiently neighbouring with equally bright pixels to satisfy the size thresholds. The number of cells on each sample was also recorded in the same manner using the same software to count the nuclei visible in each image. Dividing the total area of protein on each surface by the total number of nuclei on each surface a value was deduced for the level of protein produced per cell. This value was then made relative to a planar control composed of the same

sol-gel derived titania material. The results for the OCN protein are tabulated in Table 3. The purpose of the initial experiment was to obtain a qualitative overview of how pillar geometry would vary the differentiation of the stem cells measured by protein markers.

- 5 Table 3 - Area of OCN protein produced per cell normalised for each surface from 9 images (total area = 1447680 pixels) relative to a planar control.

| <b>Diameter (nm) →</b> | <b>40</b> | <b>70</b> | <b>100</b> |
|------------------------|-----------|-----------|------------|
| <b>Height (nm) ↓</b>   |           |           |            |
| <b>8</b>               | 0.7%      | 0.7%      | 0.7%       |
| <b>15</b>              | 3.4%      |           | 7.8%       |
| <b>20</b>              |           |           | 3.6%       |
| <b>25</b>              | 3.7%      |           |            |
| <b>30</b>              |           | 2.8%      |            |
| <b>40</b>              |           |           | 0.9%       |
| <b>60</b>              |           | 1.2%      | 0.3%       |
| <b>80</b>              | 1.2%      |           |            |

- Following the demonstration of the titania composition and integrity, the inclusion of NSQ  
 10 nanopatterns was therefore examined for osteogenic capacity [Dalby et al (2007) and (2014)]. AFM surface plots of the surfaces examined were taken and superimposed on the AFM scans was the arithmetic average roughness (Ra) measured for each sample with the standard deviation from sets of 12 samples. A polished titanium surface was found to have a surface roughness (Ra) of sub-5 nm, a planar (non-patterned) sol-gel-  
 15 derived titania surface was found to have Ra of sub-1 nm, a surface having sol-gel-derived titania NSQ nanopillars (20 nm high, 100 nm diameter) and a surface having sol-gel-derived titania NSQ nanopits (60 nm deep, 200 nm diameter were also examined).

- To optimise the osteogenic differentiation, initial work used human CD271+  
 20 osteoprogenitor cells enriched from human bone marrow. Osteogenic differentiation was

assessed by expression of bone markers, osteocalcin (OCN) and osteopontin (OPN).

100 nm diameter pillars with a height of  $19.3 \pm 1.8$  nm demonstrated enhanced osteogenesis, as indicated by Table 3. These studies confirmed translation from polymer substrates [Dalby (2007), McMurray (2011)] to a titania surface was enhanced by a  
5 change in the polarity of the pattern while NSQ geometry is preserved. Furthermore, sol-gel derived titania nanopatterns induced enhanced OPN and OCN expression in comparison to planar versions composed of the same sol-gel-derived material or untreated titanium metal (the native implant material). No correlation with surface roughness was observed. In addition, the difference in protein levels was highly  
10 significant between both nanopatterned samples and the polished planar titanium control surface with the exception of OPN on the nanopits ( $P = 0.112$ ). OCN levels were similarly raised on both pillar and pit surfaces with a noticeable enhancement in OPN following culture on nanopillars over nanopits. OPN is known to develop later in the bone formation process and the current observations may indicate a rapid stimulation of  
15 osteoprogenitors on the pillar surfaces providing an enhanced osseoinductive stimulus in contrast to nanopits.

The results of the analysis are shown in Fig. 13, which is a bar graph summarising the average amount of protein (in pixel area) produced per cell for each sample. OCN is the  
20 left hand bar and OPN the right hand bar for each surface type. Error bars indicate the standard deviation for triplicate samples. Double asterisk represent highly statistically significant ( $P$  value less than 0.01) and a single asterisk represents statistically significant ( $P$  value between 0.01 and 0.05) results with respect to the Planar Ti control.

25 In addition to osteogenic assessment using fluorescent labelling, metabolomic analysis was applied which further validated that the pillar surfaces enhanced osteogenic differentiation, as reported in the Annexes.

In summary of this section, through the synthesis of sol-gel coupled with the versatile 3D  
30 nanopatterning process, thin films of pure anatase have been realised with the capacity to

incorporate highly precise nanofeatures of either positive or negative tone. Previously the production of nanopillars via direct embossing was considered a stringent process and as a consequence biological analysis of pillar topographies had proved challenging [Gadegaard et al (2008), Greer et al (2012)]. The in vitro and in vivo studies reported  
5 here indicate that 15-20nm tall nanopillars with a diameter of 100 nm and in a disordered geometric layout provide an enhanced osteoinductive platform in comparison to planar or pitted titanium.

OPN is known to be produced by cells late in the bone formation process [Yang et al  
10 (2014) and analysis of OPN levels between nanopits and nanopillars indicated that the nanopillars expressed approximately 50% more per cell than nanopits. Ingenuity Pathway Analysis indicated that osteogenic biochemical pathways are exclusively activated on the pillar surfaces, as are mechanotransduction pathways involving integrin signalling. These empirical results are of crucial importance to the field of orthopaedic  
15 implant coatings. A translation of the presented results to clinical implants is intentionally accessible and viable offering enhanced technology delivery, uptake and healthcare outcomes as a consequence of bone growth upon orthopaedic implants. Such strategies offer tangible benefits for both the patients and healthcare sectors from potentially improved patient recovery time to enhanced implant longevity delivering desirable  
20 outcomes for an aging population.

#### Further experimental details

**Sol-gel synthesis.** All chemicals were sourced from Sigma-Aldrich. The solution is  
25 prepared by mixing 0.96 ml of diethanolamine (99%) with 5.54 ml of 1-hexanol (99%) and 0.10 ml of deionised water. The mixture should be vigorously stirred for 10 minutes before adding 3.40 ml of Ti(OBu)<sub>4</sub> (97%) while stirring. Stirring should continue in a sealed vial for 2 hours to ensure complete dissolution of the chemicals. The shelf life of this sol-gel precursor is in excess of 14 months when stored at room temperature.



**Stamp (template) fabrication.** EBL was used to define the etch mask in a bi-layer of PMMA coated Si. To produce a mould for PDMS pillars the positive-tone PMMA may itself be utilised as the etch mask, however to produce a mould for PDMS pits a metallization and lift-off is necessary to define the RIE mask. Conventional RIE may proceed [Greer (2012)]. Thereafter a trichloro silane mono-layer was deposited to facilitate compliant demoulding of PDMS. Sylgard 184 from Dow Corning was cast upon the moulds in a 1:10 / curing agent:monomer weight ratio. Once cast it was cured in an oven at 70°C overnight. Once cured the PDMS stamps containing either pits or pillars may be peeled from the master mould and trimmed with a scalpel blade.

**Imprinting.** Evaporated titanium samples were spin-coated with sol-gel at 9 krpm for 7 seconds. PDMS stamps were then placed on to the sol-gel coating, and under their own weight, corresponding to a pressure of 44 Pa, the stamp imprinted the sol-gel solution. Samples were then baked with the stamp present at 120°C for 10 minutes before removing the stamp and sintering the sample at either 500 or 700°C with a ramp rate of 2°C/min.

**STEM & EELS set-up.** Cross-section slices of the sintered sol-gel coated titanium samples were extracted using focused ion beam milling with a FEI Nova 200 Dualbeam FIB/SEM. STEM and EELS analysis was carried out using a JEOL ARM 200cf operated at 200 kV and equipped with a Gatan GIF Quantum EEL spectrometer.

**Cell culture and fluorescence microscopy.** Human CD271+ (magnetic isolation kit from Stem Cell Technologies, UK) osteoprogenitor cells were enriched from bone marrow samples donated under ethical approval by patients undergoing hip replacement surgery at Glasgow Southern General Hospital. Cells were cultured on the experimental substrates using Dulbecco's modified eagle medium supplemented with foetal bovine serum, penicillin streptomycin, non-essential amino acids, sodium pyruvate and L-glutamine (all sourced from Sigma Aldrich). The cells were cultured upon the samples for three weeks. At the end of which the samples were fluorescently tagged to identify

the cell nucleus (DAPI stain), cell cytoskeleton (actin stain) and bone related proteins OCN and OPN (antibody stain); the staining protocol deployed is explained below. To analyse the surfaces, a 3 x 3 array of images at 3 mm pitch were captured using a 10x microscope objective which gave a square field of view with side 0.79 mm. The area of protein visible in each of the nine images per sample was measured with Cell Profiler software [Carpenter (2006)]. The software parameters used were thresholds for intensity and size. The number of cells upon each sample was also recorded in the same manner using the same software to count the nuclei visible in each image. By dividing the total area of protein on each surface by the total number of nuclei on each surface a value was deduced for the level of protein produced per cell.

### Immuno-staining protocol

Component list:

- Fixative – 10 ml formaldehyde in 90 ml phosphate buffered saline (PBS) then add 2 g sucrose and dissolve.
- PBS/bovine serum albumin (BSA) = 1 g BSA in 100 ml 1 x PBS
- PBS/Tween® 20 (non-ionic detergent) = 0.5 ml Tween® 20 in 100 ml 1 x PBS
- Perm buffer = 10.3 g sucrose, 0.292 g NaCl, 0.06g MgCl<sub>2</sub> (hexahydrate), 0.476g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in 100 ml 1 x PBS. Adjust pH to 7.2 then add 0.5 ml Triton X.
- Primary antibody is diluted 1:50 parts with PBS/BSA.
- Secondary antibody is diluted 1:100 parts with PBS/BSA.
- DAPI counterstain also diluted 1:50 parts with PBS/BSA.

With the samples still inside their culture well:

- 1) Use fixative to fix cells at 37 °C for 15 mins.
- 2) Remove fixative, add perm buffer – place in a fridge at 4 °C for 5 mins.
- 3) Remove perm buffer, add PBS/BSA – keep at room temperature for 5 mins.
- 4) Remove PBS/BSA, add primary antibody. Wrap in foil and store at room temperature overnight.

- 5) Remove antibody. Wash three times for 5 mins in PBS/Tween® 20 on a plate shaker.
- 6) Remove PBS/Tween® 20 add secondary antibody. Wrap in foil and store at room temperature for 1 hr.
- 7) Remove secondary antibody. Wash three times for 5 mins in PBS/Tween® 20 on a plate shaker.
- 8) Remove sample from PBS/Tween® 20 and add DAPI counterstain. Wrap in foil and store at room temperature for 10 mins.
- 9) Remove counterstain. Wash three times for 5 mins in PBS/Tween® 20 on a plate shaker.
- 10) Place a drop of Fluoromount onto the coverslip and place the sample face down onto a coverslip.

**Metabolomics set-up and analysis.** Cells were cultured under the same conditions documented as described in “Cell culture and fluorescence microscopy”. After 14 days in culture, samples were washed in phosphate buffered saline and metabolites extracted by shaking samples in a chloroform/methanol/water solution for one hour before centrifuging at 13000g for 5 minutes at 4°C in order to remove cell debris. Metabolites were analysed using hydrophilic interaction liquid chromatography – mass spectrometry (ZIC-pHILIC (Merck Sequant) and Orbitrap Exactive (Thermo Fisher Scientific) respectively) with a 15 minute gradient running from 80% acetonitrile/20% H<sub>2</sub>O to 20% acetonitrile/80% H<sub>2</sub>O and a mass range of between 70 and 1400 Daltons in positive/negative ionisation switching mode. Metabolites were identified against mass and retention times of known standards or predicted retention time using the authentic standards as a seed using the IDEOM [Creek et al (2012)] / MzMatch [Scheltema et al (2011)] pipeline. Metabolite data was processed using IDEOM, Metaboanalyst 2.0 [Xia et al [2012) and (2009)] and IPA, Quigen (for IPA all identified metabolites were uploaded). Metabolites matched to either authentic standards or putative assignments are specified as such in the text.

**Cell extraction for in vivo experiments.** Adult human osteoprogenitor cells were obtained from the bone marrow and femoral head samples collected from

haematologically healthy patients undergoing hip replacement surgery with local ethics committee approval (LREC194/99/1), as previously described [Yang et al (2003)].

Further selection of STRO-1-enriched SSC fraction from bone marrow cell population involved using STRO-1 antibody and magnetic sorting system, as previously described

5 [Yang et al (2003), Howard et al (2002), Mirmalek-Sani et al (2006)]. SSCs were cultured in a basal medium ( $\alpha$ -MEM, Lonza/10% FCS/1% P/S) at 37°C with 5% CO<sub>2</sub>, with medium changes twice weekly. Individual experiments were carried out using cells from distinct patient donors unadjusted for demographics (5 donors for 5 repeats of in vitro experiments and 3 donors for 3 repeats of in vivo experiments). Only passage 1 cells  
10 were used. SSCs were seeded at 220/cm<sup>2</sup> density either directly onto TCP (control groups) or substrates (test groups) and cultures continued for 21 days in vitro and 28 days in vivo.

**Substrate preparation.** The substrates were sterilised for a minimum of 24 hrs in

15 PBS/1% antibiotic-antimycotic solution, Life Technologies, then transferred into culture plates and washed in PBS prior to cell seeding.

**Live/dead cell assay.** Cell viability assay was performed using CellTracker™ Green (CTG) CMFDA and ethidium homodimer-1 (Life Technologies). 50 µg of CTG and 5 µg  
20 of ethidium homodimer were dissolved in 10 µl of DMSO, and added to the culture medium.

**Immunofluorescence of OPN ex vivo.** Samples were fixed in 4% PFA, blocked with goat serum, Sigma-Aldrich, followed by overnight treatment in anti-OPN primary antibody  
25 raised in rabbit (GeneTex) followed by goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate (Life Technologies). Cells were counterstained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Life Technologies), and mounted on slides prior to imaging. Image capture was performed with Zeiss Axiovert 200 inverted microscope using an Axiovert HR camera for white light imaging and Axiocam MR  
30 camera for fluorescent imaging. Zeiss Axiovision software version 4.7 was used for

image capture and analysis. Confocal imaging was performed with Leica TCS SP5 laser scanning confocal microscope on a Leica DMI6000 inverted microscope stand using LAS-AF software.

- 5 **qPCR analysis.** Cells were released from relevant culture surfaces (8 material replicates for in vitro and 6 replicates for in vivo experiments) using Trypsin-EDTA buffer, Sigma-Aldrich, and lysed. Total mRNA extraction was performed using the Qiagen RNeasy kit according to manufacturer's instructions. mRNA samples were treated with DNase and reverse-transcribed using SuperScript first-strand synthesis system (Veriti
- 10 Thermal Cycler, Applied Biosystems). Real-time qPCR using SYBR® Select Master Mix (Life Technologies) was accomplished on 7500 Real-Time PCR system (Applied Biosystems) for expression of  $\beta$ -actin, ALP, Collagen 1, OPN and OCN genes. Primer sequences are shown in the Annexes with  $\beta$ -actin serving as the house-keeping gene. Primer sequences were validated by dissociation curve/melt curve analysis and
- 15 efficiencies of amplification for the  $\beta$ -actin primers and primers for the bone marker genes of interest were approximately equal. The comparative cycle threshold method was utilised for quantification of PCR amplification data and relative transcript levels expressed as mean  $\pm$  S.D. Data were analysed and plotted using GraphPad Prism 6 for Mac OS X software.

20

- In vivo cell culture.** Cells were labelled with PKH26 (Sigma-Aldrich), the staining reaction stopped with FCS, and the cells pelleted and washed in  $\alpha$ -MEM. The cells were analysed by flow cytometry, seeded onto appropriate substrates, and cultured in vitro for 2 days to ensure cell adherence to the substrates. The substrates were imaged prior to
- 25 implantation subcutaneously into male nude mice bilaterally (under project licence PPL 30/2880). After four weeks, implants were retrieved, substrates dissected from enveloping host tissue and further imaging undertaken. Substrates were rinsed in PBS and cells released using Trypsin-EDTA buffer (Sigma-Aldrich), resuspended in PBS and sorted on a FACSAria™ II cell sorter (BD Biosciences) to separate original PKH26

stained fluorescent human SSC fraction from mouse (host) cells. Cells were pelleted, lysed and examined for qPCR of bone marker gene expression as described above.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention.

- 10 All references referred to above and/or identified below are hereby incorporated by reference.

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**CLAIMS**

1. A method of forming nanostructures on a curved surface of a substrate, the method including:
  - 5 providing a conformable template having template nanostructures,  
providing a sol-gel comprising a patterning precursor,  
applying the conformable template to the curved surface with the sol-gel interposed between the conformable template and the curved substrate to form a patterned coating of patterning precursor on the curved substrate, and
  - 10 subsequently carrying out thermal treatment of the patterned coating in order to develop the nanostructures.
2. A method according to claim 1 wherein the Young's modulus of the material of the conformable template is not greater than 30 MPa.
- 15 3. A method according to claim 1 or claim 2 wherein the template nanostructures of the conformable template are formed using a master substrate.
4. A method according to claim 3 wherein electron beam lithography (EBL) is used
- 20 to fabricate the master substrate.
5. A method according to any one of claims 1 to 4 wherein the material for the conformable template is polydimethylsiloxane (PDMS).
- 25 6. A method according to any one of claims 1 to 5 wherein the patterning precursor comprises at least one material which is, or which is transformable to (e.g. by thermal treatment) metal oxide, such as titanium oxide.
7. A method according to any one of claims 1 to 6 wherein the sol-gel comprises an
- 30 alcohol having a vapour pressure at 20°C of not more than 50 mmHg.

8. A method according to any one of claims 1 to 7 wherein the patterning precursor comprises a metal-based alkoxide.

5 9. A method according to any one of claims 1 to 8 wherein the sol-gel comprises diethanolamine, 1-hexanol, deionised water and  $\text{Ti}(\text{OBu})_4$ .

10. A method according to any one of claims 1 to 9 wherein the sol-gel is coated onto at least one of the conformable template or onto the substrate.

10

11. A method according to any one of claims 1 to 10 wherein the conformable template and the substrate are pressed together with a pressure of not more than 2 bar.

12. A method according to any one of claims 1 to 11 wherein the assembly of  
15 conformable template, sol-gel and substrate held together and the sol-gel is cured, the conformable template then being removed from the substrate.

13. A method according to any one of claims 1 to 12 wherein the thermal treatment to develop the nanostructures achieves at least partial sintering of the material of the  
20 nanostructures.

14. A method according to any one of claims 1 to 13 wherein the nanostructures comprise titania, and after the thermal treatment the titania is predominantly of the anatase phase.

25

15. A method according to any one of claims 1 to 14 wherein the substrate is formed of at least 50% by mass of a material with Young's modulus of at least 50 GPa, ultimate tensile strength (UTS) of at least 100 MPa and fracture toughness  $K_{Ic}$  of at least 10  $\text{MPa}\cdot\text{m}^{-0.5}$ .

30

16. A method according to any one of claims 1 to 15 wherein the substrate is metallic.

17. A method according to any one of claims 1 to 16 wherein the nanostructures on the curved surface of the substrate are arranged in a pattern based on a notional  
5 symmetrical lattice in which the distance between nearest neighbour notional lattice points is C, between 10 nm and 10  $\mu$ m, and the nanostructures are locally mis-ordered such that the centre of each topographical feature is a distance of up to one half of C from its respective notional lattice point.

10 18. A method according to any one of claims 1 to 17 wherein the nanostructures are protrusions and/or recesses.

19. A method according to any one of claims 1 to 18 wherein the nanostructures are pillars of diameter 40-100nm and height 8-80nm.

15

20. A medical implant with at least one curved surface, the curved surface having a patterned array of metal oxide nanostructures formed on it.

20

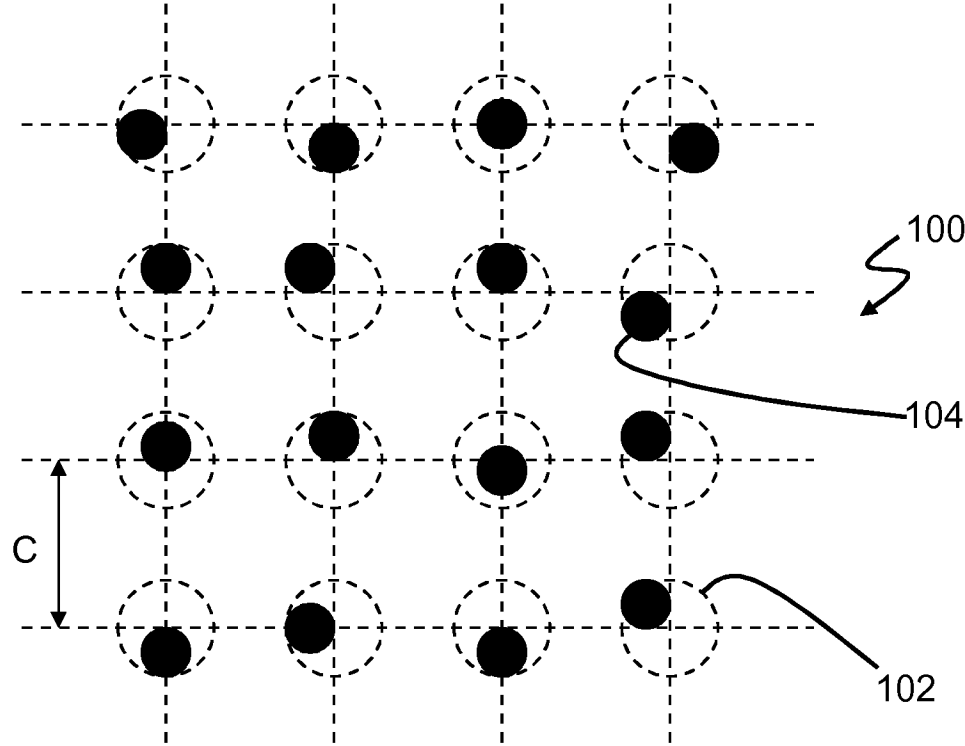


Fig. 1

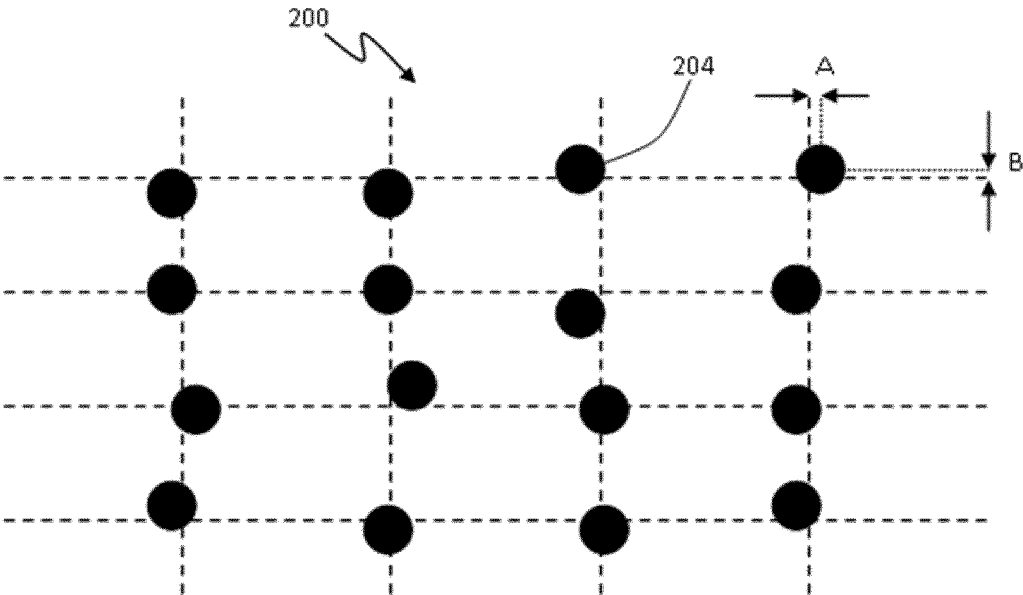


Fig. 2

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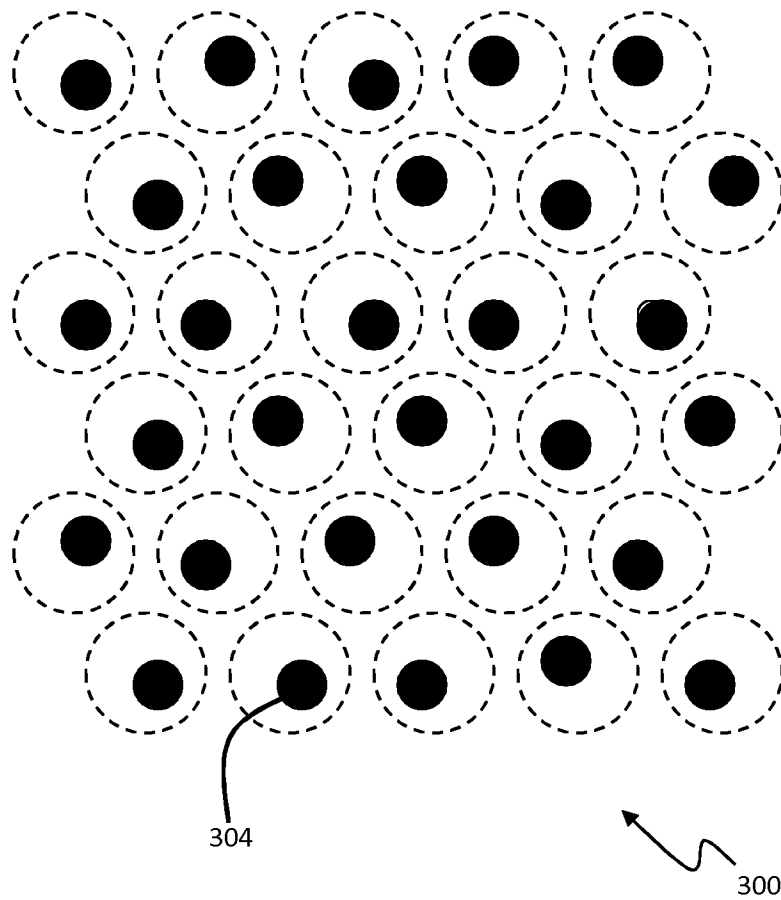


Fig. 3

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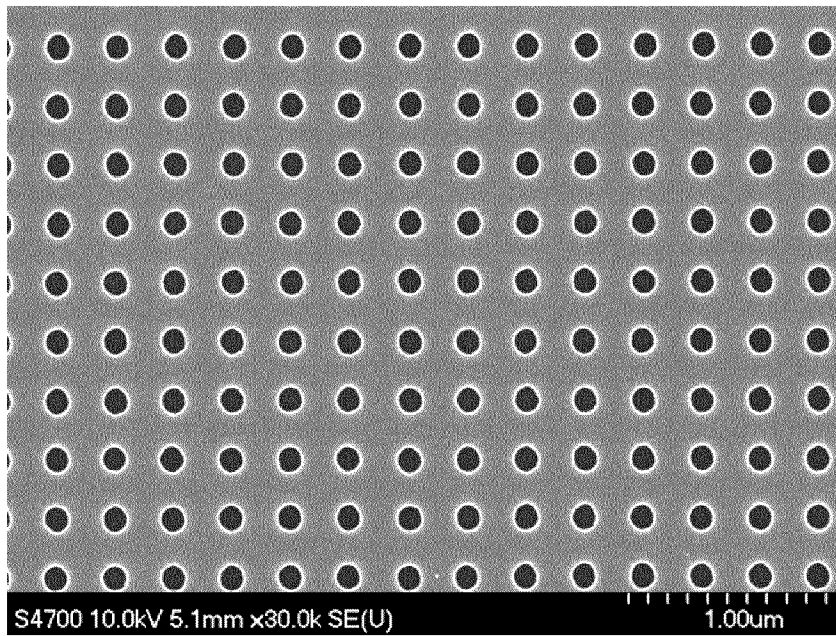


Fig. 4

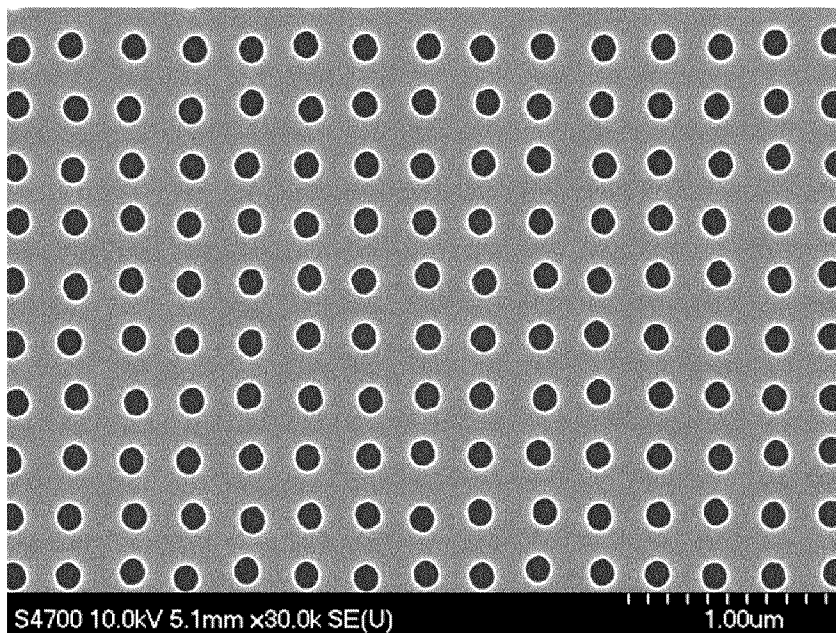


Fig. 5



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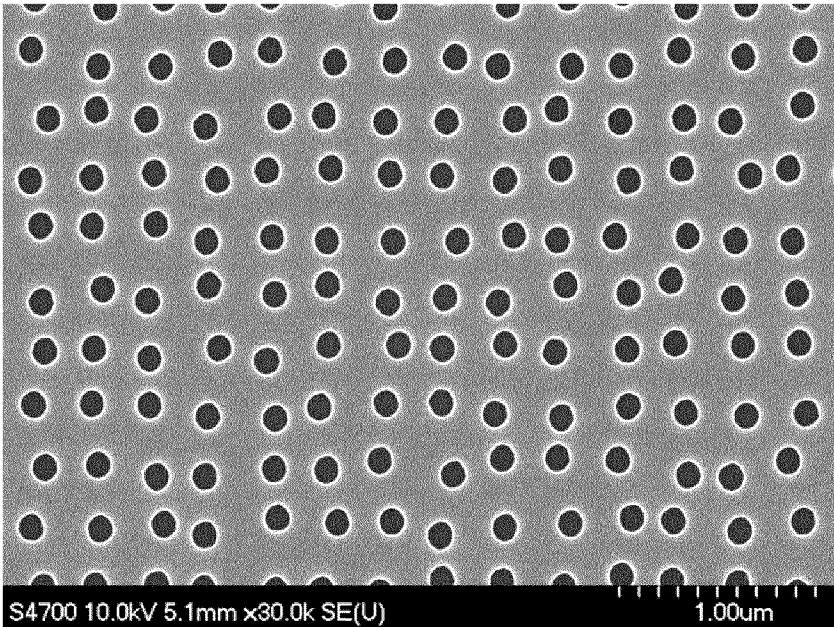


Fig. 6

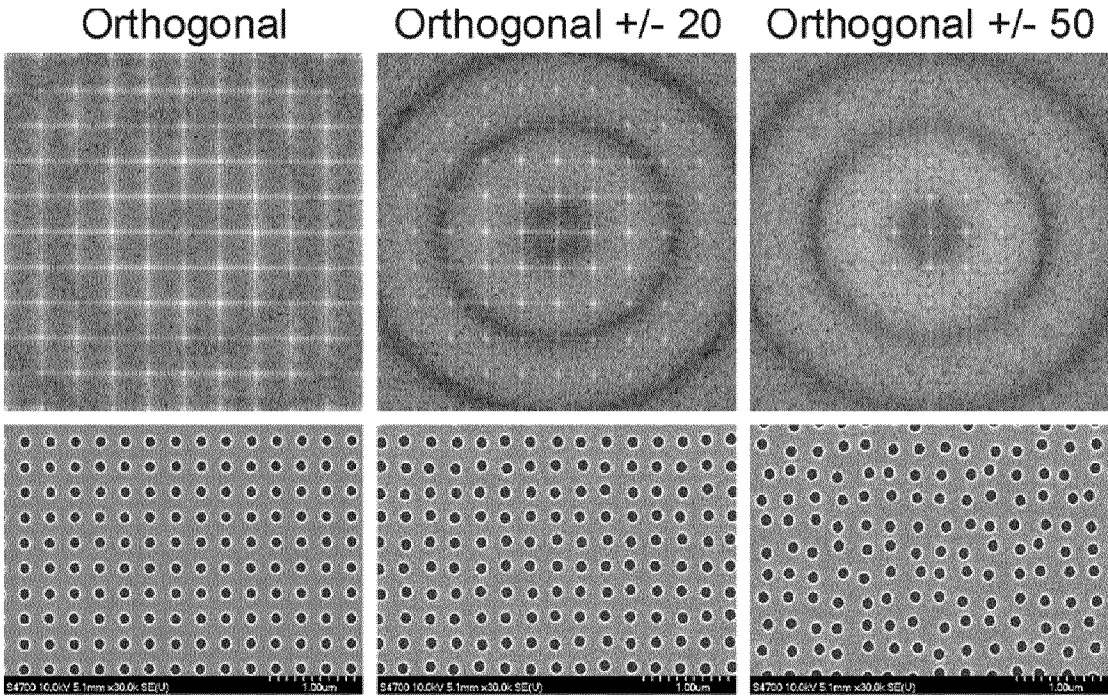


Fig. 7

1 µm

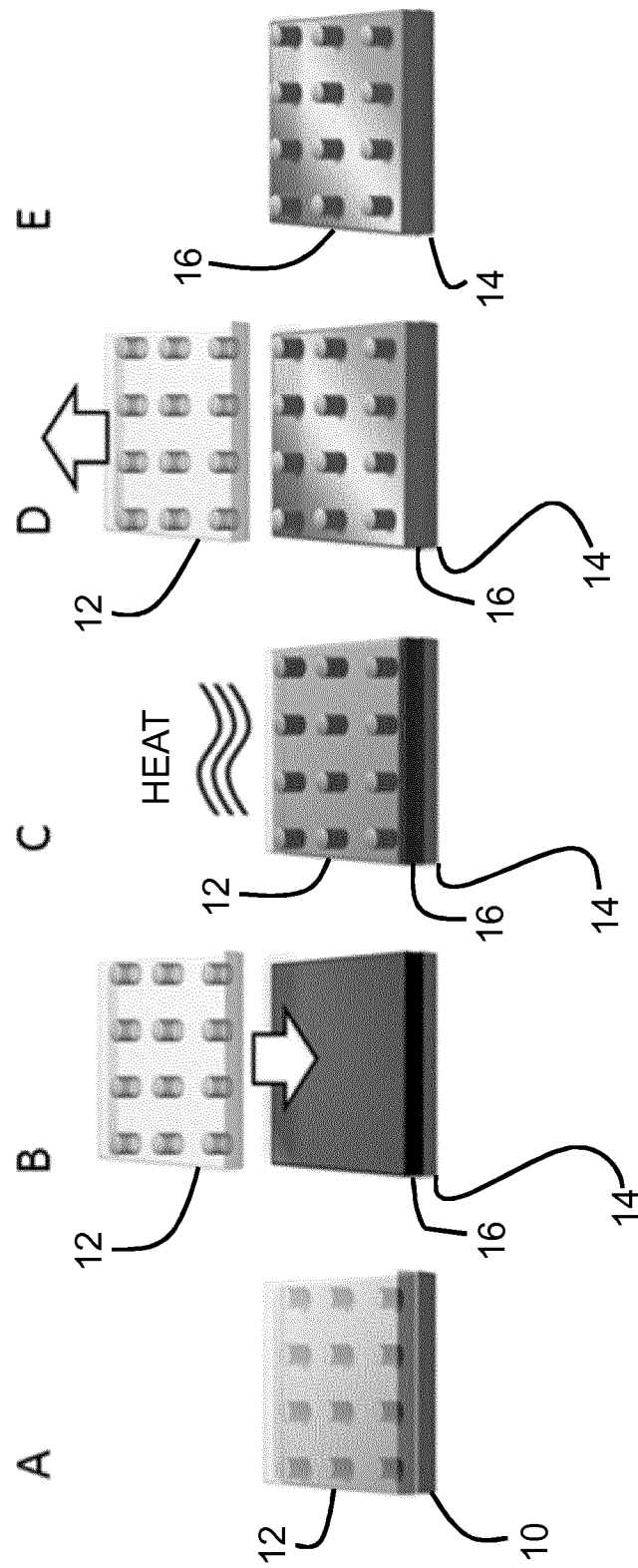


Fig. 8

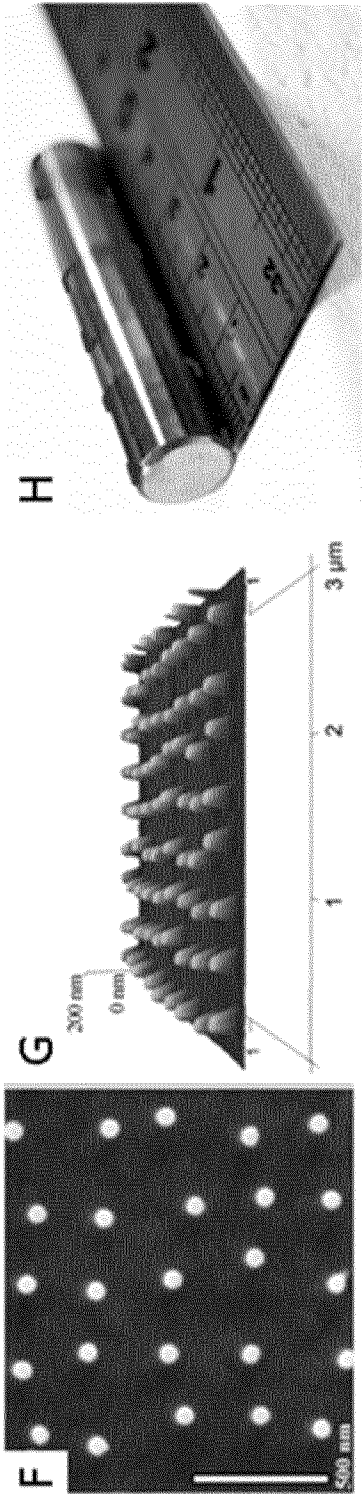


Fig. 9

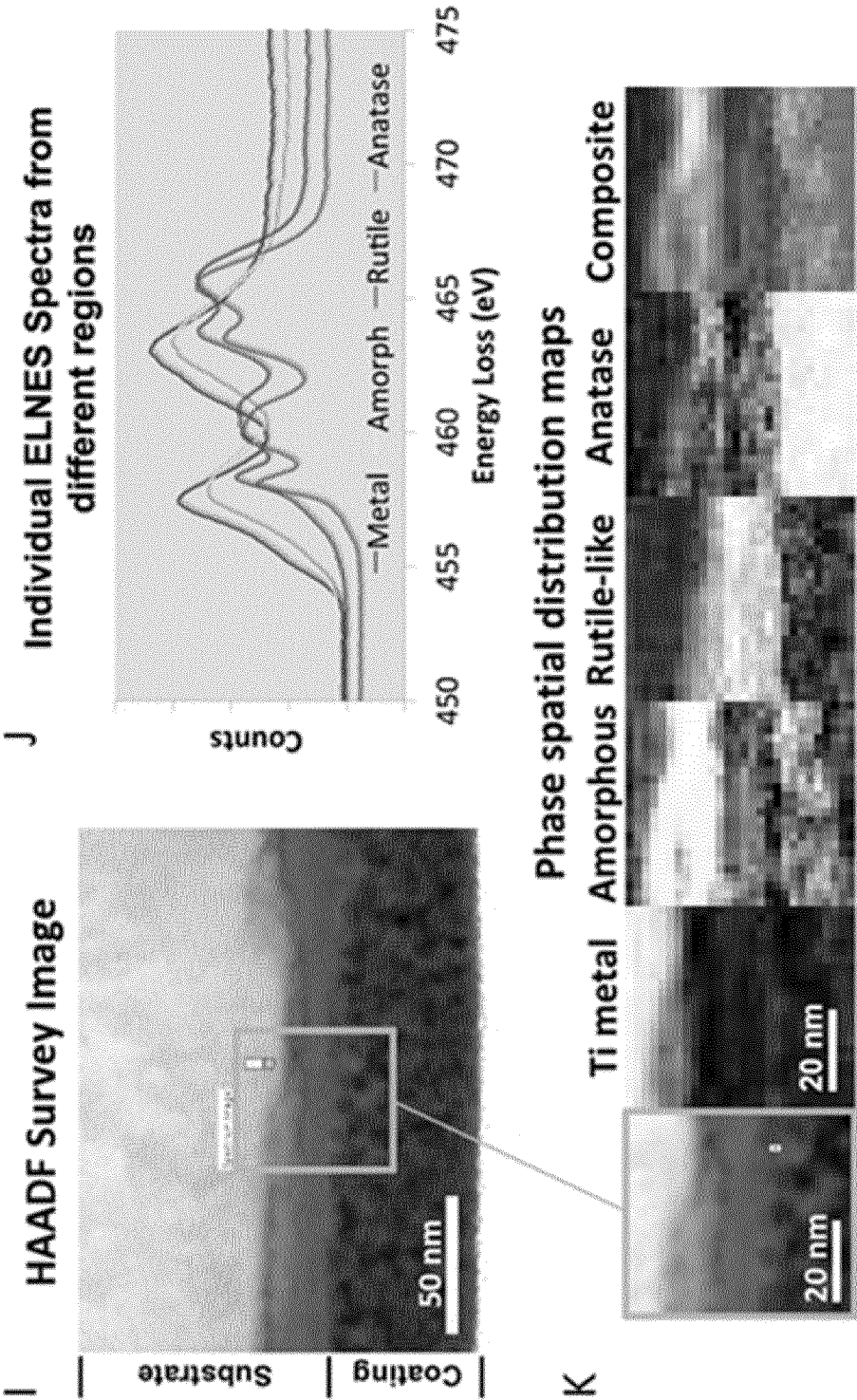
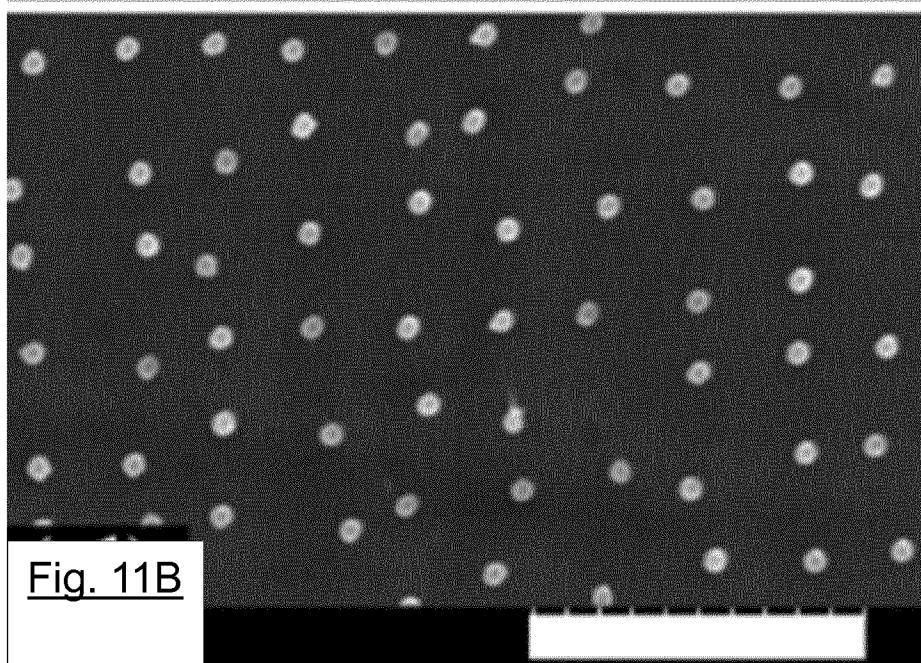
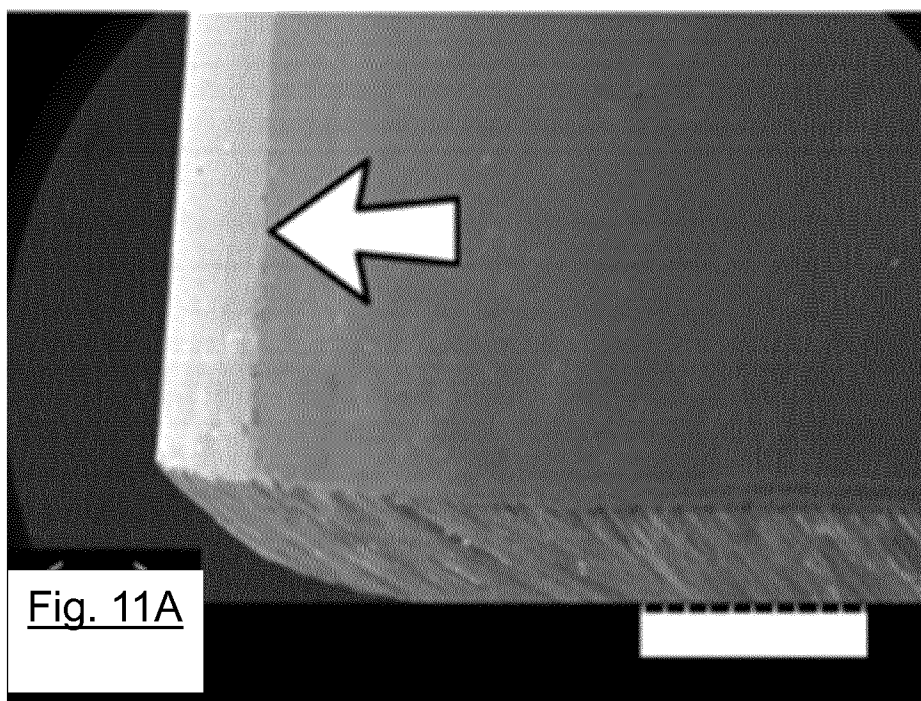


Fig. 10

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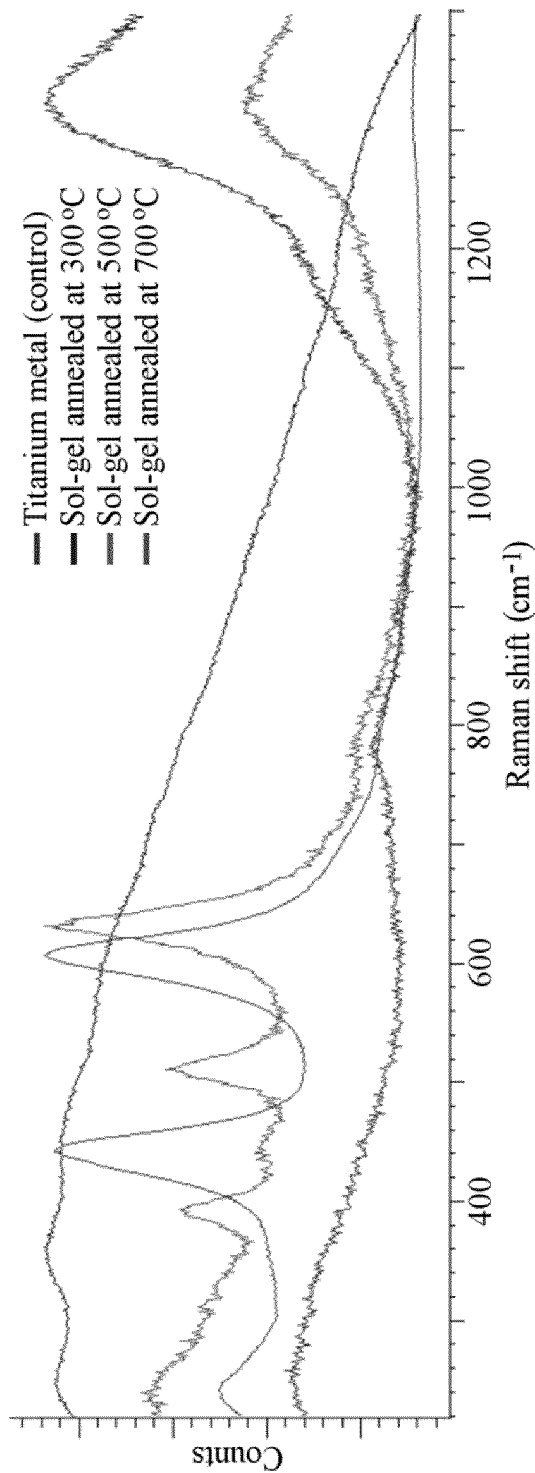


Fig. 12

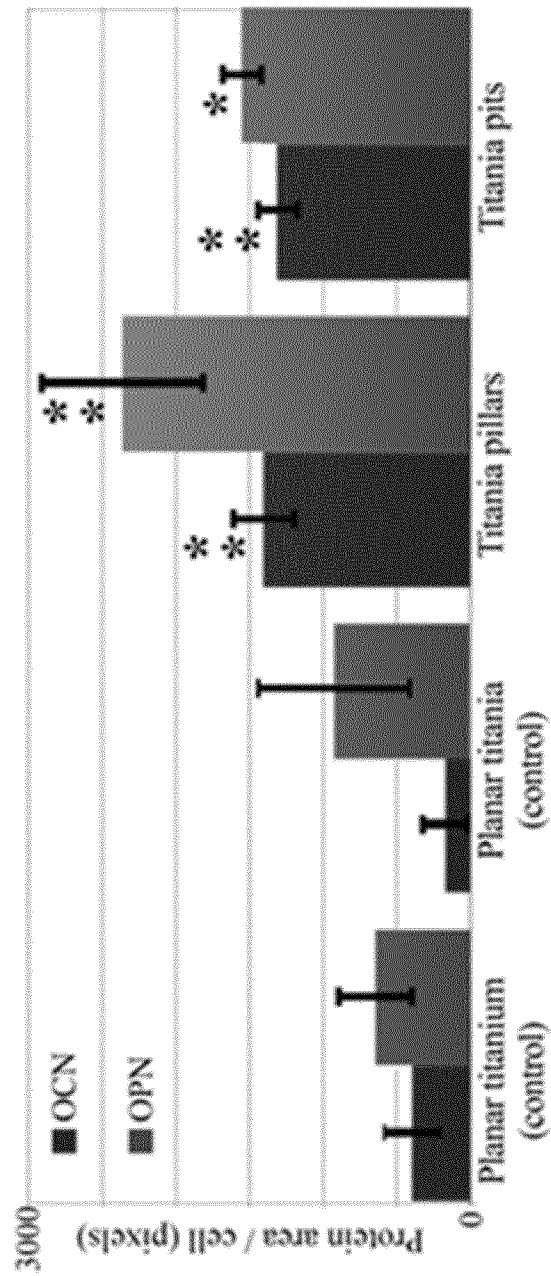


Fig. 13



## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2016/082254

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C23C18/12 A61L27/30  
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)  
C23C 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

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                        | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------|-----------------------|
| X         | EP 2 878 977 A1 (FOM INST FOR ATOMIC AND MOLECULAR PHYSICS [NL])<br>3 June 2015 (2015-06-03)              | 1-6,<br>10-19         |
| Y         | paragraph [0032] - paragraph [0033];<br>figure 1A<br>paragraph [0065] - paragraph [0073]<br>-----<br>-/-- | 7-9                   |



Further documents are listed in the continuation of Box C.



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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

16 February 2017

Date of mailing of the international search report

23/02/2017

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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2016/082254

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                     |                       |
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## INTERNATIONAL SEARCH REPORT

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