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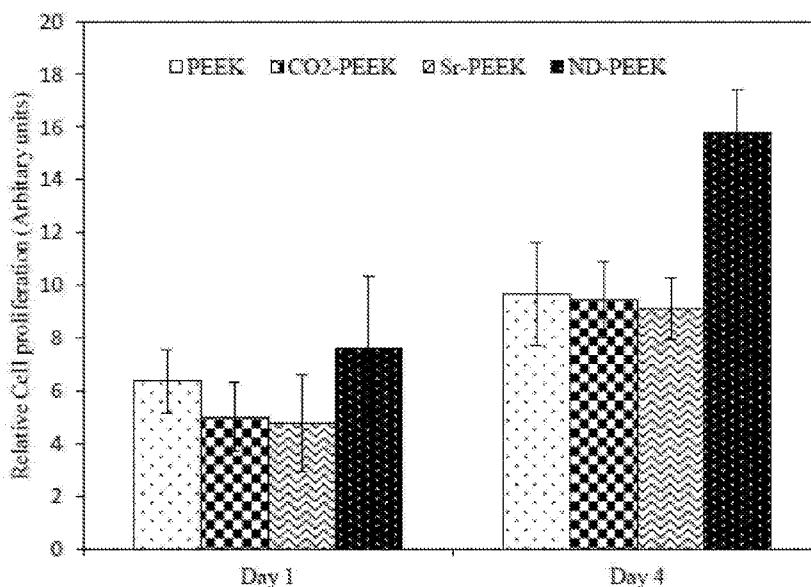


FIG. 21

(57) Abstract: The invention relates to the incorporation of bone-growth reactive compounds and anti-infection agents upon the surface of an implantable polymeric material to enhance the biological properties of the resultant multifunctional composite material. To this end, supercritical carbon dioxide was used to plasticise the surface of the polymer and permit the incorporation of bone-bonding compounds and anti-microbial compounds to the structure of the implants. Nanodiamond particles and a Tea Tree Oil-derived compound, terpinen-4-ol, were used to enhance the bone bonding and antimicrobial properties of the implants, respectively.



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BIOMEDICAL IMPLANTABLE MATERIALS

Field of the Invention

The present invention relates to developments in biomedical applications. More particularly, the invention relates to the high pressure impregnation of the surface of polyether ether ketone (PEEK) for implantable devices, such as orthopaedics. PEEK inherently exhibits good mechanical properties for orthopaedic implants – and the present invention seeks to enhance the latter two categories.

Although the present invention will be described hereinafter with reference to its preferred embodiments, it will be appreciated by those of skill in the art that the spirit and scope of the invention may be embodied in many other forms.

Background of the Invention

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Surgical implants offer a long-term solution to bone and joint damage, but are notorious to failure due to surgical, biological and mechanical causes. Two factors that may lead to implant failure are the lack of implant-to-bone bonding ability and the high risk of infection at the implantation site. Accordingly, a serious challenge faced by bone tissue engineers is the design of a biomaterial that can successfully integrate with bone tissue by mimicking the natural function of bone; another is the design of a biomaterial exhibiting antimicrobial properties.

Polyether ether ketone (PEEK) is a material of choice for the fabrication of bone and joint implants due to its superior mechanical strength (its bulk mechanical properties are close to those of human bone), benign biochemical composition – and its radioactive and chemical stability. On the other hand, the major limitation of PEEK is its lack of bioactivity (*i.e.*, bioinertness – the bone bonding ability of a material under *in vivo* conditions) that leads to limited fusion with bone when placed into the body. A secondary limitation is PEEK's lack of antibacterial capacity.

The bulk mechanical properties of PEEK are close to that of human bone, making it a suitable orthopaedic implant material. Various *in vitro* and *in vivo* studies have suggested that this material is biocompatible. However, the bio-inert nature of PEEK has delayed its

biological interaction with host tissues [Toth, J.M., *et al.*, 2006. *Biomaterials*, 27(3): p. 324-334; and Lui, S.-c. *et al.*, 2003. *Biomaterials*, 24(26): p. 4871-4879].

Despite the superior properties that make this material an outstanding candidate for orthopaedic biomaterial design, PEEK has a major limitation. Its chemical inertness render it non-bioactive leading to limited fusion/fixation with bone when placed into the body. This has been a major concern associated with PEEK biomedical implants. Therefore, it requires an external material to act as an interphase between the bone and PEEK implant. Another disadvantage is its limited ability to tailor mechanical properties for a particular implant design. Increasing effort has been directed to tackling the above disadvantages and subsequently improving the bone implant interface by producing composites with HA, by coating PEEK implants with Ti and HA and by creating porous PEEK networks for bone in-growth.

In respect of PEEK, it is difficult to obtain a working balance between its biological properties and mechanical properties. There exists a design trade-off between increased bioactivity with increased levels of calcium phosphate reinforcement, and decreased strength. Patent publications of note are WO 2011/072212 and WO 2014/152649, both assigned to Diffusion Technologies, Inc., and WO 2014/151016, to Blackstone Medical, Inc.

It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative. It is an object of preferred forms of the present invention to provide a means of: a) incorporating bioactive compounds at the interface of the PEEK so as to enhance the biological profile of the resultant modified material; or b) to impregnate PEEK with an antibacterial compound, thereby to mitigate the risk of infection at the implantation site. As such, for the purposes of the ensuing description, the invention will be described separately with respect to each application.

Accordingly, the present invention relates broadly to modifying the surface of PEEK to incite enhanced bioactivity and favourable cell responses, both of which are prerequisites for successful material integration with living bone tissue. Early reaction of body fluids with implants is critical for subsequent cell interaction and eventual material-tissue integration. In one form, nanodiamonds are used to enhance the biological surface properties of PEEK. Nanodiamonds have been shown to elicit enhanced bioactive and cell adhesion properties. In another form, terpinen-4-ol (derived from the Australian native Tea Tree Plant) is currently attracting attention as a compound that may alleviate the predisposition of implant devices to failure due to its antimicrobial properties. Accordingly, the present invention also relates to

the fabrication and efficacy of multifunctional PEEK surfaces developed by using supercritical CO₂ to incorporate nanodiamond and terpinen-4-ol.

Definitions

5 Throughout the description, examples and claims, the experimental parameters under which the surface of PEEK is plasticised generally include the use of high pressure, supercritical carbon dioxide at a temperature of 80 °C; a pressure of 180 bar; and a reaction time of 6 h. It will be appreciated that literal adherence to these parameters is not essential in order to effectively plasticise the PEEK surface (in turn, making it amenable to the
10 impregnation of additive materials, such as nanodiamonds or terpenin-4-ol). Indeed, any variation of the quoted parameters that will give rise to the desired technical effect of surface plasticisation is within the ambit of the present invention.

In respect of temperature, any variation within the general range of 31 to 100 °C is considered within the scope of the present invention. For example, 31, 32, 33, 34, 35, 36, 37,
15 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100 °C are all contemplated, as are intermediary values such as 70.5, 71.5, 72.5, 73.5, 74.5, 75.5, 76.5, 77.5, 78.5, 79.5, 80.5, 81.5, 82.5, 83.5, 84.5, 86.5, 87.5, 88.5, and 89.5 °C.

20 In respect of pressure, any variation within the general range of 73 to 200 bar is considered within the scope of the present invention. For example, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138,
25 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199 and 200 bar are all contemplated, as are intermediary values such as 170.5, 171.5, 172.5, 173.5, 174.5, 175.5, 176.5, 177.5, 178.5, 179.5, 180.5,
30 181.5, 182.5, 183.5, 184.5, 185.5, 186.5, 187.5, 188.5 and 189.5 bar.

The skilled person will also appreciate that the relationship between temperature and pressure for carbon dioxide is easily ascertainable from a phase diagram; these abound on the internet.

In respect of time, any variation within the general range of 0.2 to 48 h is considered within the scope of the present invention. For example 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8, 5, 5.2, 5.4, 5.6, 5.8, 6, 6.2, 6.4, 6.6, 6.8, 7, 7.2, 7.4, 7.6, 7.8, 8, 8.2, 8.4, 8.6, 8.8, 9, 9.2, 9.4, 9.6, 9.8, 10, 10.2, 10.4, 10.6, 10.8, 11, 11.2, 11.4, 11.6, 11.8, 12, 12.2, 12.4, 12.6, 12.8, 13, 13.2, 13.4, 13.6, 13.8, 14, 14.2, 14.4, 14.6, 14.8, 15, 15.2, 15.4, 15.6, 15.8, 16, 16.2, 16.4, 16.6, 16.8, 17, 17.2, 17.4, 17.6, 17.8, 18, 18.2, 18.4, 18.6, 18.8, 19, 19.2, 19.4, 19.6, 19.8, 20, 20.2, 20.4, 20.6, 20.8, 21, 21.2, 21.4, 21.6, 21.8, 22, 22.2, 22.4, 22.6, 22.8, 23, 23.2, 23.4, 23.6, 23.8, 24, 24.2, 24.4, 24.6, 24.8, 25, 25.2, 25.4, 25.6, 25.8, 26, 26.2, 26.4, 26.6, 26.8, 27, 27.2, 27.4, 27.6, 27.8, 28, 28.2, 28.4, 28.6, 28.8, 29, 29.2, 29.4, 29.6, 29.8, 30, 30.2, 30.4, 30.6, 30.8, 31, 31.2, 31.4, 31.6, 31.8, 32, 32.2, 32.4, 32.6, 32.8, 33, 33.2, 33.4, 33.6, 33.8, 34, 34.2, 34.4, 34.6, 34.8, 35, 35.2, 35.4, 35.6, 35.8, 36, 36.2, 36.4, 36.6, 36.8, 37, 37.2, 37.4, 37.6, 37.8, 38, 38.2, 38.4, 38.6, 38.8, 39, 39.2, 39.4, 39.6, 39.8, 40, 40.2, 40.4, 40.6, 40.8, 41, 41.2, 41.4, 41.6, 41.8, 42, 42.2, 42.4, 42.6, 42.8, 43, 43.2, 43.4, 43.6, 43.8, 44, 44.2, 44.4, 44.6, 44.8, 45, 45.2, 45.4, 45.6, 45.8, 46, 46.2, 46.4, 46.6, 46.8, 47, 47.2, 47.4, 47.6, 47.8 and 48 hours are all contemplated, as are intermediary values such as 5.1, 5.3, 5.5, 5.7, 5.9, 6.1, 6.3, 6.5, 6.7 and 6.9 hours.

Thus, whilst certain preferred embodiments of the present invention are described using an experimental temperature of 80 °C; a pressure of 180 bar; and a reaction time of 6 h, the skilled person will readily appreciate that in order to practise the invention, literal compliance is not essential.

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising” and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

Although the invention will be described with reference to specific examples it will be appreciated by those skilled in the art that the invention may be embodied in many other forms.

Nomenclature

For the sake of familiarity and convenience, the materials prepared during experimental work toward the present invention have been named in an informal, yet descriptive, manner; such nomenclature is adhered to throughout the ensuing description and

examples. The following non-exhaustive list is provided, along with their respective rationalisations.

Table 1 – Examples of generic nomenclature used throughout the present invention

Nomenclature	Description
Naked PEEK	“Naked” = untreated, <i>i.e.</i> , polyether ether ketone (PEEK), having unaltered surface characteristics.
PEEK-CO ₂	PEEK, having had its surface plasticised by supercritical CO ₂ .
Sr-PEEK	PEEK, having had its surface plasticised and impregnated with strontium-doped phosphate glass particles.
ND-PEEK	PEEK, having had its surface plasticised and impregnated with nanodiamonds.
PEEK-son-ND	PEEK, having had its surface plasticised and impregnated with nanodiamonds; and then having been sonicated.
PEEK-TTO	“TTO” = Tea Tree Oil, <i>i.e.</i> , PEEK, having had its surface plasticised and impregnated with terpinen-4-ol.
PEEK-TTO-6h	PEEK, having had its surface plasticised and impregnated with terpinen-4-ol over a 6 hour period.
PEEK-TTO-24h-Eth	PEEK, having had its surface plasticised and impregnated with terpinen-4-ol over a 24 hour period; and then washed with ethanol.

5

Summary of the Invention

The invention relates generally to the incorporation of bone-growth reactive compounds and anti-infection reagents upon the surface of the PEEK to enhance the biological properties of this class of hard tissue implants. To this end, supercritical CO₂ was used to plasticise the surface of the PEEK and permit the incorporation of bone-bonding compounds and anti-microbial compounds onto the structure of the implants. Nanodiamond particles and terpinen-4-ol were used respectively to enhance the bone bonding and antimicrobial properties of the PEEK implants.

According to a first aspect of the present invention, there is provided a method for enhancing the biomedical profile of an implantable material, the method comprising the steps of: selecting the implantable material; subjecting the implantable material to a first treatment step comprising exposure to supercritical carbon dioxide, at a first predetermined temperature, under a first predetermined pressure, over a first predetermined time, thereby to plasticise at least a portion of the surface of the implantable material; subjecting the plasticised surface of the implantable material to a second treatment step, performed concurrently with or subsequent to the first treatment step, the second treatment step comprising exposure to an additive material in a medium of supercritical carbon dioxide, at a second predetermined temperature, under a second predetermined pressure, over a second predetermined time, thereby to impregnate at least some of the additive material into at least

a portion of the plasticised surface of the implantable material, thereby to provide a composite implantable material.

In a preferred embodiment, the implantable material comprises polyether ether ketone (PEEK). In another preferred embodiment, the implantable material is comprised of,
5 or consists essentially of polyether ether ketone (PEEK).

In another embodiment, the inventive method further comprises performing a post-treatment washing step, thereby to remove at least some non-embedded, or non-chemically-associated particles of the additive material (*i.e.*, unreacted additive material). In another embodiment, the biomedical profile of the implantable material is enhanced with respect to
10 the biocompatibility of the composite material; or the resistance of the composite material to bacteria.

In another embodiment, the first predetermined temperature is between about 31 and about 100 °C, the first predetermined pressure is between about 73 and about 200 bar and the first predetermined time is between about 0.2 hours and about 48 hours, more preferably,
15 between about 0.2 and about 24 hours. More preferably, the first predetermined temperature is about 80 °C, the first predetermined pressure is about 180 bar and the first predetermined time is about 6 hours.

In another embodiment, if the second treatment step is not performed concurrently with the first treatment step, the second predetermined temperature is between about 31 and
20 about 100 °C, the second predetermined pressure is between about 73 and about 200 bar and the second predetermined time is between about 0.2 hours and about 48 hours, more preferably, between about 0.2 and about 24 hours. More preferably, the second predetermined temperature is about 80 °C, the second predetermined pressure is about 180 bar and the second predetermined time is about 6 hours.

25 In another embodiment, the inventive method further comprises the step of polishing the surface of the implantable material prior to undertaking the first treatment step. In another embodiment, the composite implantable (surface-modified) material exhibits bulk thermal and mechanical properties that are not reduced to a statistically-significant degree with respect to those of the untreated implantable material.

30 In one especially preferred embodiment, the biomedical profile of the implantable material is altered by enhancing the biocompatibility of the material. Preferably, this is achieved wherein the additive material is a plurality of nanodiamonds. In another embodiment, during the second treatment step, the nanodiamonds are provided as a

suspension in an alcoholic carrier prior to being subjected to the supercritical carbon dioxide. Preferably, the alcoholic carrier comprises ethanol. Most preferably, the alcoholic carrier comprises ethanol in a concentration of about 70% to about 100%; it was found that beyond about 30% concentration, water could adversely impact upon the aggregation of the nanoparticles.

In another embodiment, the step of providing the nanodiamonds in a suspension of ethanol comprises pasting the suspended nanodiamond particles onto the surface of the implantable material prior to subjecting the reactant materials to the second treatment step comprising supercritical carbon dioxide impregnation. In another embodiment, the suspension of nanodiamonds in ethanol has a solids content of between about 5 mg/mL and about 100 mg/mL. More preferably, the suspension of nanodiamonds in ethanol has a solids content of about 10 mg/mL.

In another embodiment, the inventive method further comprises a sonication step, thereby to reduce any agglomeration of the nanodiamonds suspended in the ethanol. Preferably, the sonication step is defined by parameters comprising a period of about 2 h; an output control setting of 500 W, 20 kHz; and a constant duty cycle.

In another embodiment, the biocompatibility of the material is enhanced by the impregnation of nanodiamonds upon at least a portion of the surface of the implantable material, the impregnated nanodiamonds providing a platform for the fusion of a hydroxyapatite (HA) layer with the composite surface, the HA layer subsequently providing a platform to enhance bone mineralisation. Preferably, the HA layer also serves to enhance osteoblast adhesion and proliferation.

In another embodiment, the surface roughness average parameter R_a of the untreated implantable material reduces after the first treatment step, and reduces further after the second treatment step. In another embodiment, the surface roughness average parameter R_a for nanodiamond-impregnated PEEK, having undergone about 1 hour of post-treatment sonication is between about 10 and about 20 nm. More preferably, the surface roughness average parameter R_a for nanodiamond-impregnated PEEK, having undergone about 15 min of post-treatment sonication is between about 10 and about 50 nm, most preferably, as determined empirically, about 14.2 nm.

In another embodiment, the post-treatment washing step employs distilled water, followed by sonication in ethanol for about 15 minutes, thereby to remove any excess or non-impregnated (*i.e.*, unreacted) nanodiamond particles from the surface of the composite

material. In another embodiment, the nanodiamond-impregnated PEEK samples show a relatively uniform distribution of particle-like structures upon the surface of the treated PEEK.

5 In another embodiment, the individual particle-like structures have an average height of between about 10 and about 40 nm. In another embodiment, the nanodiamond-impregnated PEEK exhibits an increased contact angle in wettability tests relative to untreated PEEK, said increased contact angle indicative of the increased hydrophobicity of the composite surface.

10 According to a second aspect of the present invention, there is provided an implantable material having a biomedical profile enhanced by a method as defined according to the first aspect of the present invention, wherein the additive material is a plurality of nanodiamonds.

15 In one preferred embodiment, the impregnation of nanodiamonds upon the surface of the implantable material does not significantly alter the bulk thermal mechanical properties of the implant for load-bearing applications, thereby ensuring the required functional matching to hard bodily tissue. In another embodiment, the modified implantable material couples the favourable bio-response enhancing profile of nanodiamonds with the advantageous thermal mechanical properties of PEEK, thereby to render the composite material amenable for use as a bone biomaterial.

20 In an alternative embodiment, the biomedical profile of the implantable material is altered by enhancing the resistance of the material to bacteria. Preferably, the additive material is terpinen-4-ol. In another embodiment, as an optional precursor step, the terpinen-4-ol is extracted from Tea Tree Oil using supercritical carbon dioxide. Alternatively, the terpinen-4-ol is sourced commercially.

25 In another embodiment, the step of providing the terpinen-4-ol comprises pasting about 50 μ L of terpinen-4-ol onto the surface of the implantable material prior to subjecting the reactant materials to the second treatment step comprising supercritical carbon dioxide impregnation. In another embodiment, the washing step comprises ethanol, thereby to wash away terpinen-4-ol that is not chemically associated with the surface of the composite material (*i.e.*, unreacted) following the second treatment step with supercritical carbon
30 dioxide.

In another embodiment, the composite terpinen-4-ol-impregnated PEEK exhibits greater hydrophobicity after supercritical CO₂ treatment than does untreated PEEK. In

another embodiment, the inventive method further comprises the step of employing a slow depressurisation rate using 100 nm diameter capillary tubing following the first and/or second treatment steps.

In another embodiment, the composite material of terpinen-4-ol-impregnated PEEK shows resistance to *S. aureus* and *P. aeruginosa* for incubation time points of 10 minutes, 1 h and 24 h.

According to a third aspect of the present invention, there is provided an implantable material having a biomedical profile enhanced by a method as defined according to the first aspect of the present invention, wherein the additive material is terpinen-4-ol.

In an especially preferred embodiment, the impregnation of nanodiamonds upon the surface of the implantable material does not significantly alter the bulk thermal mechanical properties of the implant for load-bearing applications, thereby ensuring the required functional matching to hard bodily tissue. In another embodiment, the modified implantable material couples the favourable antibacterial-enhancing profile of terpinen-4-ol with the advantageous thermal mechanical properties of PEEK, thereby to render the composite material amenable for use as an implantable material that may inhibit bacterial attachment during the initial stages of implant surgery.

According to a fourth aspect of the present invention, there is provided an implantable composite material having nanodiamonds impregnated within at least a portion of the surface area of the implantable material, the nanodiamond-impregnated implantable material thereby having an enhanced bone bonding ability (integration with bone) relative to an equivalent material without the impregnated nanodiamonds.

According to a fifth aspect of the present invention, there is provided an implantable composite material having terpinen-4-ol impregnated within at least a portion of the surface area of the implantable material, the terpinen-4-ol-impregnated implantable material thereby having an enhanced antibacterial profile relative to an equivalent material without the impregnated terpinen-4-ol.

According to a sixth aspect of the present invention, there is provided a method of performing orthopaedic surgery, said method comprising implanting at a surgical site a material as defined according to the second aspect of the present invention, or the fourth aspect of the present invention.

According to a seventh aspect of the present invention, there is provided a method of performing orthopaedic surgery, said method comprising implanting at a surgical site a

material as defined according to the third aspect of the present invention, or the fifth aspect of the present invention.

According to an eighth aspect of the present invention, there is provided use of an implantable composite material as defined according to the second aspect of the present invention, or the fourth aspect of the present invention, in orthopaedic surgery.

According to a ninth aspect of the present invention, there is provided use of an implantable composite material as defined according to the third aspect of the present invention, or the fifth aspect of the present invention, in orthopaedic surgery.

The first form of the invention focused on incorporating a bone-bonding promoting effect by using 4-5 nm hydrophobic nanodiamond particles. Much work went into optimising the impregnation of nanodiamonds onto the PEEK before any cellular and apatite studies could take place. The optimum conditions for impregnation which were previously determined were at 180 bar, 80 °C and 6 h. Surface changes undergone due to supercritical impregnation were not significant, such as the decrease in nano-roughness of 18 to 16 nm, enhanced uniformity of thermal mechanical property distributions and a decrease in hydrophobic characteristic for fabricated PEEK incorporated with nanodiamond (PEEK-son-ND). No significant changes were observed in total surface free energy as a result of supercritical CO₂ exposure. The cellular viability study results indicate favourable effects of PEEK-son-ND to human osteosarcoma cells MG-63 by having an initial count of 1500 cells leveraged over the naked PEEK surface (day 1). Consistent increased cellular activity was seen through to days 5 and 8 before hitting a plateau as cells reached confluence. Thirty minute simulated body fluid tests produced visual confirmations of aggregated apatite formation on PEEK-son-ND. Interestingly, naked PEEK attracted formation of singular spherical apatite on uniform areas of the surface.

Surface plasticisation of the PEEK was achieved using a high pressure CO₂-based technology – and the nanoparticles or biomolecules were then embedded on the modified surface. The inventive process appears to occur only in conditions that are specific to the polymer. The impregnated compounds are incorporated only into the very top layer of the PEEK (to less than 200 nm; the depth is controllable by the process parameters) and provided for as yet unreported surface functionality. Structural and chemical analyses demonstrated a uniform distribution of the impregnated particles. Each of the tested particles significantly upregulated bioactivity (*i.e.*, bone-bonding ability) of the PEEK; *in vitro* bioactivity has been demonstrated using both the standardised method ISO/FDIS 23317 and

cell-based assays. Furthermore, bioactivity has been confirmed by the precise analysis of apatite formation ability assay using atomic force microscopy [Chrzanowski, W., *et al.*, 2012. *RSC Advances*, Volume 2, p. 9214-9223] and cell-based assays.

This part of the investigation was subdivided in to four sections: (i) optimisation of temperature parameter to plasticise PEEK using high pressure carbon dioxide; (ii) separate incorporation of nanodiamonds and strontium-doped phosphate glass particles into different PEEK samples using high pressure carbon dioxide; (iii) studying the effect of new surface modification on bioactivity; and (iv) *in vitro* studies to examine the effect of the new surface modification on cell behaviour.

Evidence of impregnation was affirmed by the appearance of small particle-like structures on PEEK surface and apparent variations in nanoscale surface roughness. However, *in vitro* studies demonstrated that specific nanoscale surface roughness did not elicit any influence on cellular activity

Water contact angle measurements revealed that the new surface modifications imparted hydrophobic properties to both ND-PEEK (nanodiamond-impregnated PEEK) and Sr-PEEK (strontium-doped phosphate glass particle impregnated PEEK). ND-PEEK had the highest water contact angle measurement of 94°, followed by Sr-PEEK with 89.9° and PEEK with 78.58°. Evaluation of surface free energy revealed a slightly different pattern with ND-PEEK having the highest surface free energy (40.3 mN/m) and Sr-PEEK (34.87 mN/m) having the lowest surface free energy. In spite of the increased hydrophobicity, the most favourable bioactivity and cellular responses were demonstrated by ND-PEEK. This was attributed to the inherent property of nanodiamonds to facilitate enhanced growth of hydroxyapatite in SBF and improve osteoblast adhesion and proliferation.

The bioactivity of newly modified ND-PEEK and Sr-PEEK were evaluated by immersing the samples in simulated body fluid (SBF) for up to 24 h. The surface of both ND-PEEK and Sr-PEEK were covered by a continuous layer of bone-like apatite in just 6 h, suggesting that both modifications were effective in inducing fast bioactivity in simulated body fluid conditions. However the surface of ND-PEEK showed fastest precipitation (in less than 1 h) of bone-like apatite. Moreover, the sizes of the precipitates formed by ND-PEEK were comparatively larger, indicative of higher bioactivity potential.

In vitro cell activity studies showed that both surface modifications supported the adhesion and proliferation of osteoblasts. As expected, ND-PEEK demonstrated very high cellular activity, but the cellular activity observed on Sr-PEEK was relatively poor in

comparison. The results suggest that the inventive surface modification of PEEK with nanodiamonds has the potential to create a bioactive interface that can establish effective bonding between the living bone tissue and the biomaterial, thereby improving implant integration within the body. It therefore has the potential to be used as a biomaterial in bone tissue engineering applications.

Further work fabricated an antimicrobial model implant surface with the incorporation of derived terpinen-4-ol from an Australian native plant, *Melaleuca alternifolia* (Tea Tree Oil) upon the plasticised PEEK surface. Much work went into optimising the fabrication before any antibacterial studies could take place. Optimisation of fabrication was employed by using two sets of variables, 35 and 80 °C and 24 and 6 h. Optimisation results obtained from wettability, surface free energy, FTIR-ATR characterisations and thermal-mechanical analyses revealed that temperature and time variation did not improve the deposition of terpinen-4-ol onto the surface of PEEK. FTIR-ATR confirmation of the chosen fabrication parameters (80 °C, 180 bar and 6 h) with an approximate loading of 50 µL terpinen-4-ol per sample was successful by detection of characteristic alcohol, aromatic and hydrocarbon bonds found on the IR spectra as an indication of terpinen-4-ol presence on the fabricated sample surface. Supercritical CO₂ treatment effects were not significant except with the change in the sample's surface average roughness profile as reflected by the decrease of the roughness average parameter R_a to 10 nm from 18 nm. Cell viability studies revealed loading of 50 µL terpinen-4-ol per active sample surface area induced toxicity to human cell line MG-63. Impaired bacterial attachment and adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was achieved on the fabricated PEEK surface (37 °C incubation time point of 10 minutes).

The main technique used for surface modification is high pressure carbon dioxide technology. With PEEK being an amorphous polymer, it was expected that using high pressure carbon dioxide would be a feasible technique to incorporate nanodiamonds and strontium-doped phosphate glass particles. The high pressure nature of carbon dioxide has the capability to impregnate even a non-reactive penetrant into the polymer matrix. This was achieved by plasticisation of the polymer, upon which nanodiamonds can be incorporated. The subsequent depressurisation that follows ensured that the nanodiamonds remained within the polymer matrix. The effect of carbon dioxide on the surface of different PEEK samples was tested by adjusting variables such as temperature from around 40 °C to 80 °C, in order to obtain the ideal conditions for plasticising the PEEK surface. The tests used to

characterise the surface of the PEEK sample employed atomic force microscopy (AFM).

Next, the biomimetic bone-like growth of hydroxyapatite on the surface of PEEK in the presence of nanodiamond was studied by placing the new sample in SBF for a predetermined period of incubation (6 h, 12 h and 24 h) using AFM techniques. The samples were monitored every few hours for the growth of HA layer. Finally, cell viability and proliferation were assessed using an alamarBlue® assay. Scanning electron microscopy and AFM are then employed to image the morphology of the cells.

Brief Description of the Figures

A preferred embodiment of the invention will now be described, by way of example only, with reference to the accompanying drawings in which:

Figure 1 is a phase AFM image (2 x 2 μm) of PEEK-son-ND distinguishing contrasts such as blackened regions for the relatively stiff nanodiamond and lighter regions for softer material, *i.e.*, PEEK.

Figure 2 shows height AFM images (5 x 5 μm) of: (A) ND-PEEK without sonication; and (B) PEEK-son-ND.

Figure 3 depicts 3D AFM images of: (A) ND-PEEK without sonication; and (B) PEEK-son-ND.

Figure 4 is a plot of the thermal mechanical property profile for: (A) PEEK-CO₂; and (B) PEEK-son-ND for heating temperatures of 52.48 to 235.48 °C.

Figure 5 plots the cell count for naked PEEK (solid), PEEK-CO₂ (horizontal scores), PEEK-son-ND (checkered) and cell control/media (hollow) at days 1, 5 and 8; the results are presented as mean ($\pm$ standard error).

Figure 6 shows AFM imaging (20 x 20 μm) for naked PEEK: (A) naked PEEK in 30 minutes SBF height; (B) naked PEEK in 30 minutes SBF amplitude; and (C) naked PEEK in 30 minutes SBF phase.

Figure 7 shows AFM imaging (20 x 20 μm) for PEEK-CO₂: (A) PEEK-CO₂ in 30 minutes SBF height; (B) PEEK-CO₂ in 30 minutes SBF amplitude; and (C) PEEK-CO₂ in 30 minutes SBF phase.

Figure 8 shows AFM imaging (20 x 20 μm) for PEEK-son-ND: (A) PEEK-son-ND in 30 minutes SBF height; (B) PEEK-son-ND in 30 minutes SBF amplitude; and (C) PEEK-son-ND in 30 minutes SBF phase.

Figure 9 are 3D AFM images corresponding with the AFM images shown in *Figure*

19, above: (A) naked polished PEEK; (B) PEEK 80 °C.

Figure 10 shows line profiles of the PEEK samples subjected to different temperature treatments for optimisation: (A) naked polished PEEK; and (B) 80 °C.

Figure 11 is an amplitude AFM image of ND-PEEK.

5 Figure 12 is a 3D AFM image corresponding to the ND-PEEK sample depicted in Figure 25, above.

Figure 13 is a line profile of the ND-PEEK sample shown in Figures 25 and 26, above.

Figure 14 shows the water contact angle of PEEK (dotted black), PEEK-CO₂ (checkered), Sr-PEEK (waved lines) and ND-PEEK (dotted white) samples and their relative significance.

Figure 15 shows the surface free energies and their components for PEEK and modified PEEK samples.

15 Figure 16 shows AFM images of samples incubated in SBF for 6 h: (A) PEEK; and (B) ND-PEEK.

Figure 17 are the 3D AFM images corresponding with the AFM images shown in Figure 30, of samples incubated in SBF for 6 h: (A) PEEK; and (B) ND-PEEK.

Figure 18 shows the line profile of selected samples incubated in SBF for 6 h: (A) PEEK; and (B) ND-PEEK.

20 Figure 19 shows AFM images of samples incubated in SBF for 1 h: (A) amplitude image of PEEK only; and (B) amplitude image of ND-PEEK.

Figure 20 depicts 3D AFM images of selected samples incubated in SBF for 1 h: (A) PEEK; and (B) ND-PEEK.

25 Figure 21 shows the alamarBlue® assay results, demonstrating cell proliferation on days 1 and 4 for: PEEK (dotted black), PEEK-CO₂ (checkered), Sr-PEEK (waved lines) and ND-PEEK (dotted white) samples.

Figure 22 shows the Fourier Transform Infrared with Attenuated Total Reflectance crystal spectrographs of: (A) pure terpinen-4-ol solution; (B) terpinen-4-ol present on PEEK-TTO-6h.

30 Figure 23 is the 3D AFM image of PEEK-TTO-6h.

Figure 24 shows mean values of the water contact angle ($\pm$ standard error) for the surfaces of: naked PEEK (diagonal scores), PEEK-TTO-24h (dotted) and PEEK-TTO-24h after ethanol washing (solid).

Figure 25 depicts the mean values of the total surface free energy (dotted; $\pm$ standard error), comprising its polar (diagonal lines) and dispersive (checkered) components for naked PEEK, PEEK-TTO-6h, PEEK-TTO-6h after ethanol washing, PEEK-TTO-24h and PEEK-TTO-24h after ethanol washing.

5 Figure 26 shows the thermal mechanical property profile for PEEK-TTO-24h, for heating temperatures of 52.48 to 235.48 °C.

Figure 27 depicts the cell count for: naked PEEK (solid), PEEK-TTO-6h (diagonal lines) and cell control/media (hollow) at days 1, 5 and 8; the results for cell count are presented as mean ($\pm$ standard deviation).

10 Figure 28 shows SEM micrographs of: (A) 10-minute specimen showing a naked PEEK sample occupied by *P. aeruginosa* (white arrows); and (B) 10-minute specimen showing a PEEK-TTO-6h sample occupied by *P. aeruginosa* (yellow arrows); the distinctive destruction of the microbial structure observed; scale indicated by red line on lower left (2 μ m).

15 Figure 29 is a comparative pair of SEM micrographs for: (A) 10-minute specimen showing a naked PEEK sample occupied by *S. aureus* (white arrows); the “grape-like” bunch appearance of *S. aureus* is indicative of healthy cell-to-cell adhesion and interaction; and (B) 10-minute specimen showing a PEEK-TTO-6h sample occupied by *S. aureus* (yellow arrows); the rounded singular appearance of *S. aureus* is indicative of dying microbes; scale indicated by red line on lower left (20 μ m).

20

Experimental

Plasticisation of the polymer surface – high pressure CO₂ impregnation of PEEK

This technique involves the use of carbon dioxide at a temperature and pressure above its critical point. Carbon dioxide is a commonly-used substance because of its relatively low critical temperature ($T_c = 31.1$ °C) and pressure ($P_c = 73.8$ bar), which makes it a feasible way to process thermo-labile compounds.

25

Impregnation

30 Impregnation is usually carried out to bring about a modification in the polymer properties, it involves three steps: 1) exposing the polymer to high pressure carbon dioxide for a period of time; 2) introduction of carbon dioxide containing solutes to the polymer and the transfer of solute from carbon dioxide to the polymer; and 3) release of carbon dioxide in

a controlled manner and trapping the solute in the polymer.

Platform for implant design

There are three main types of implant applications: applications in soft tissue, hard
5 tissue and prosthetics. Different applications have different material property requirements.
For instance, harder tissues require higher strength material with good wear properties and a
flexibility that matches that of the hard tissues. Soft tissues require low strength materials
that are relatively more flexible than those employed in hard tissue applications.
Biocompatibility is also different for each application as it requires some degree of potential
10 bone integration ability in hard tissue applications to succeed.

Mechanical properties

In hard tissue applications, the interest is primarily focused on mechanical matching.
It is important to match elastic modulus and tensile strength with the tissue involved, *e.g.*,
15 bone or teeth. Such applications usually employ implants with load-bearing capabilities.
Tensile strength is important; matching the load bearing capacity of the hard tissue affords
long-term durability. Also, higher tensile strength is equivalent to having better wear
properties which alleviates occurrences of inflammation in a subject.

Chemical, thermal and radiation stability; biocompatibility

The chemical stability of a material is crucial when exposed to an aqueous 37 °C host
environment. Materials are designed to endure long-term exposure to the large amounts of
chemicals present in the biological environment. It should also be able to withstand
exposure to ethylene oxide as a disinfecting agent. The thermal effects of sterilisation, which
25 occurs as high pressure steam, deteriorate the tensile properties of some materials.

The biocompatibility of implants covers a wide range of biological performance
indicators which may be essential, depending upon the end purpose of the implant. It is
generally required that materials be non-toxic, non-mutagenic, non-carcinogenic and non-
immunogenic.

30

Types of implant material

Within the context of the present invention, polymers are widely used in medical
applications such as implantation and drug delivery. They are largely dependent upon time

and crystallinity, which in turn determine its mechanical properties. Polymers are easily available and vary in composition, properties and forms. However, the common problem in the use of polymers is that they are too flexible and mechanically weaker than metals, which often calls for some reinforcement (discussed below). They are also prone to leaching fillers, plasticisers, antioxidants and absorbing some liquids, depending upon the nature of the application.

Antimicrobial activity

As noted above, biological or chemical solutions may have improved bioactivity on implant surfaces for hard tissue applications as a result of different methods. Most physiochemical methods involve the alteration of surface energy, surface charge and surface composition.

ND-PEEK (nanodiamond impregnated PEEK) for cellular and apatite modulation

This phase of the investigation relates to fabricating nanodiamond-functionalised PEEK using supercritical CO₂ impregnation – and to investigate cellular and apatite modulation in response to the changes resulting from the impregnation. Supercritical CO₂ impregnation was carried out under the parameters mentioned above, by using nanodiamond suspensions in ethanol with a solids content of 100 mg/mL. Additional sonication was employed after discovering the agglomerated state of these nanoparticles in ethanol. The final concentration was reduced to 10 mg/mL and the addition of sonication for 1 h, using an output control setting of 30 and a constant duty cycle, was found necessary to achieve optimum impregnation. Impregnation method validation was achieved using phase contrast imaging on AFM.

Cell viability studies were undertaken in order to determine cell metabolic behaviour towards the fabricated PEEK surface. The final part of this study investigated the ability of the modified surface to encourage bone-like hydroxyapatite formation in the presence of simulated *in vivo* conditions. Results from cell and apatite studies confirmed that correlations were able to be drawn between the changes in surface characteristics and heightened biological response. The suitability of the fabricated PEEK for use in biomedical implants will depend on the potential for positive cellular response and enhanced apatite modulation upon contact with body fluids.

Table 2 – Mean size diameter, % volume and size polydispersity index (PDI) of diamond nanoparticles in ethanol solution.

Sample	1st Step (h, treatment)	2nd Step (h, treatment)	3rd Step (h, treatment)	Peak 1 size (d.nm)	% Volume Peak 1	Peak 2 size (d.nm)	% Volume Peak 2	PDI
"N1"	-	-	-	5560 ± 1132.1	100	-	-	0.127
"N2"	1, SO	-	-	502.2 ± 142.5	88.6	155.5 ± 37.53	11.4	0.881
"N3"	1, SO	24, ST	-	503.2 ± 201.8	51.1	4972 ± 865.8	48.9	0.26
"N4"	1, SO	48, ST	0.5, SO	1722 ± 527.8	100	-	-	0.103

Results for average size are presented as mean ± standard deviation (n=1)

"SO" = solution with nanodiamond particles was sonicated using microtip;

"ST" = nanodiamond particles were left in ethanol under static conditions

Stability of diamond nanoparticles

The aim of this part of the study was to investigate the stability of diamond nanoparticles in ethanol under different static and dynamic conditions. ZetaSizer analysis revealed an overall reduced size polydispersity and agglomeration of diamond nanoparticles in ethanol with the addition of microtip sonication. *Table 2*, above, shows changes in the mean diameter and the proportions of nanoparticles of different sizes with the addition of sonication. Samples N1 to N4, all composed of 0.1 % w/v (0.02 g in 2 mL) nanodiamond in ethanol, were treated under different sonication regimes in order to optimise particle dispersion. The preliminary state of the particles in ethanol, Sample N1 in *Table 2*, was found to be monodispersed with a mean particle size of approximately $5.6 \pm 1.1 \mu\text{m}$. The particles were found to have been broken down with the addition of 1 h sonication, leaving a small proportion in a resulting $155 \pm 0.38 \text{ nm}$ mean diameter and the larger proportion at approximately $502 \pm 143 \text{ nm}$. With Sample N3, it was found that leaving the solution overnight after 1 h sonication encouraged agglomeration. Results for Sample N4 revealed that adding the extra 30 minutes sonication after nominated treatments was not sufficient in breaking down larger agglomerates formed over the two day period of rest.

Effect of sonication-supplemented nanodiamond impregnation on PEEK surface topography

Atomic force microscope measurements were performed primarily to confirm the presence of embedded nanodiamond particles on the modified PEEK surface and secondly to investigate the changes made to the surface topography after supercritical CO₂ exposure under high pressure and temperature conditions.

Figure 1 shows that for the sample supplemented with sonication PEEK-son-ND, there were easily-distinguishable stiffer components indicated by the dark grey spots while white identified with a more flexible material. Phase imaging for ND-PEEK without sonication proved to be invaluable as the image showed similar shades of dark grey in all regions without contrast to background indicating confluent high stiffness throughout the surface.

The occurrence of contrast on *Figure 1* confirmed the presence of nanodiamonds on PEEK-son-ND which were thought to be the dark grey spots due to its higher stiffness compared to the PEEK substrate. The lack of contrast in ND-PEEK imagery is suggested to be due to aggregated nanodiamonds heavily covering the surface of ND-PEEK; this gives rise to visible evidence of successful impregnation as large particles may be sitting on top of each other. The clear visual comparison that can be made between ND-PEEK and PEEK-son-ND is that both surfaces show different feature sizes and heights present when scanned under AFM. *Figures 2(A) and 2(B)* show height images of ND-PEEK and PEEK-son-ND surfaces, respectively where white spots are indications of taller objects relative to the surface of PEEK (which is the grey-black background). From *Figure 2(A)*, large aggregates of $> 1 \mu\text{m}$ having a collective height of nearly 300 nm were observed on the surface ND-PEEK while from *Figure 2(B)*, PEEK-son-ND had finer particles and more uniformly dispersed impregnation on its surface. *Figures 3(A) and 3(B)* show supplementary imaging of the different topographical features on the surfaces ND-PEEK and PEEK-son-ND, respectively.

Effect of supercritical CO₂ impregnation on PEEK topography and morphology

R_a is a height-based, arithmetic average of the absolute values of the roughness profile ordinates. The results obtained from Gwyddion showed that the roughness average parameter R_a for both naked PEEK reduced after supercritical CO₂ exposure, nanodiamond impregnation and even more so with the addition of 1 h sonication for PEEK-son-ND (naked PEEK 18.2; PEEK-CO₂ 17.8; ND-PEEK 17.9; PEEK-son-ND 14.2 nm). The roughened surface of ND-PEEK managed to produce a relatively higher R_a compared to all treated samples, while PEEK-son-ND produced the lowest indicating that the impregnation had a smoothening effect on the surface. However, these resulting values translate to no significant statistical changes in R_a .

Effect of supercritical CO₂ impregnation on surface thermal mechanical properties

It is essential to retain the bulk thermal mechanical properties of the implant for load-bearing applications to ensure the required functional matching to hard tissue is met [Hench, L. L. and Jones, J. R., 2005. *Biomaterials, Artificial Organs and Tissue Engineering*. Cambridge: Woodhead Publishing Limited]. These nano-thermal analyses (Nano-TA) show changes in thermal mechanical property distribution over a 10 x 10 µm surface of a sample. Results shown in *Figures 4(A)* and *4(B)* reveal that there were no significant changes to the surface thermal-mechanical properties as a result of supercritical CO₂ impregnation. Overall, the sampling curves obtained were uniform throughout all three samples (naked PEEK, PEEK-CO₂ and PEEK-son-ND, respectively) indicating that the method of fabrication managed to preserve the treated sample's surface structural integrity.

Wettability

The sessile droplet technique was used to determine the changes in surface free energy on the fabricated PEEK surface as a result of impregnation. Calculations of the surface free energy showed that PEEK had a hydrophobic character. Water droplet tests following impregnation resulted in a decrease in PEEK surface hydrophobicity. However, the water contact angle results revealed that there were small changes in hydrophobicity to the samples as an increase in hydrophilic character was observed.

Cell viability on fabricated PEEK surface

According to studies [Vandrovcová, M. and Bacáková, L., 2011. *Physiological Research*, 60(3), pp. 403-17], cells are found to favour proliferation in the presence of carbon nanoparticles. It is crucial that the fabricated PEEK-son-ND demonstrates this effect to confirm the viability of its application in biomedical implants. The results of *in vitro* cell viability study are presented in graphical form as cell count through days 1, 5 and 8, as presented in *Figure 5*. The first observation that could be made was that there was an initial decrease in cell count for all samples in day 1 from control of 1×10^5 cells in the media.

The day 1 cell count decrease indicated that not all cells managed to attach during seeding (day "0"). Coincidentally, although the increase in cell proliferation observed supplemented claims of studies mentioned above, there is a possibility that this result was not largely influenced by the insignificant decrease in nanoscale roughness reported above. The likely primary influence in the attachment and proliferation of cells was attributed to the

attraction of cells to the carbon found in nanodiamonds on the surface. However, the quantification of nanodiamonds impregnated on the surface cannot be determined at this stage; an extensive model to determine the toxic limit to cells may be developed in future studies.

5

Apatite formation on fabricated PEEK surface

Collective images of control samples and samples after 30 minutes simulated body fluid (SBF) immersion are presented in *Figures 6(A) to 6(C)*, *Figures 7(A) to 7(C)* and *Figures 8(A) to 8(C)*. It was found that the surface of samples on naked PEEK samples, 10 apatite started to grow uniformly across entire surface area and had the typical spherical form (*Figure 6*), while on PEEK-CO₂ samples, only very small amounts of apatite were observed (*Figure 7*). Interestingly, PEEK-son-ND was found to be composed of predominantly apatite aggregates in some regions. Detection of these aggregates was confirmed visually for different regions of the sample surface. These aggregates appeared to 15 have had a “popcorn-like” structure and were distributed non-uniformly.

It can be surmised that the growth of these aggregates was induced by the nanodiamond. Negatively charged nanodiamond particles attract calcium ions which act as seeds – and thus, the growth of apatite was induced in this structure. There are limited data available on early time point apatite detection at 30 minutes. Thus, other SBF-based studies 20 would not be an accurate basis for comparison. Collective comparisons show that increased apatite formation on PEEK-son-ND was attributable to the presence of nanodiamonds on the surface in contact with SBF.

Materials and methods applied

25 PEEK-Optima were obtained from *Invisio* biomaterials solutions (Pennsylvania, USA). This was then cut into circular shaped samples of 1 cm diameter and 2 mm thickness. The surfaces of these samples were then polished to a mirror finish using RE 4000 paper. The samples were then washed thoroughly in distilled water and dried with pressurised air, prior to surface modification. Food grade carbon dioxide (99% purity) was supplied by 30 BOC. The strontium-doped phosphate glass particles and nanodiamond used in the study were mixed separately with 70% alcohol to form a thick slurry and stored in sealed containers prior to surface modification. The nanodiamond particles were obtained from Ray Techniques Ltd (Israel). Silicon AFM tips with diamond-like carbon coating (Tap150DLC)

having a force constant of 5 N/m were used for image scanning; these were obtained from TED PELLA Inc (California, USA). Human osteosarcoma cell line (MG-63) was cultured and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum under humidified atmosphere of 5% CO₂ at 37 °C. The alamarBlue® was
5 obtained from ABD Serotech (USA). PBS (phosphate buffered saline) and media were obtained from Sigma Aldrich Company Ltd. Naked cleaned PEEK samples were used as control.

Supercritical carbon dioxide impregnation of PEEK

10 Samples weighing approximately 250 g were placed in a 100 mL Thar high pressure vessel, with the mirror finish surface facing on the top. The temperature of the system was allowed to increase to the desired temperature (variations of 40 °C, 60 °C and 80 °C). The vessel was then sealed off and pressurised to 180 bar, using a syringe pump (ISCO, Model 500D). The system was then maintained at the desired temperature and pressure for a fixed
15 period of reaction time (6 h), after which the CO₂ pressure was rapidly vented off to the atmosphere, the vessel opened and the samples removed, following which the sample weights were recorded. The effects of CO₂ impregnation at the varying conditions were further studied by measuring surface roughness and observing the surface topography using AFM. The optimal conditions were used for further modification of PEEK.

20

Surface topography and roughness

The surface topography of all samples was studied using AFM (MFP-3D). All samples were scanned under air topography mode, using a silicone probe coated with diamond-like carbon. Five magnifications were used to study surface topography namely
25 x 20 µm, 10 x 10 µm, 5 x 5 µm, 2 x 2 µm and 1 x 1 µm. The roughness was evaluated from line profiles on the images. The magnification used for line profiles varied for different stages.

Bioactivity – SBF assays

30 SBF solution comprising Solution A and Solution B, having the chemical composition as listed in Table 3 was prepared using the method outlined in a previous study [Bohner, M. and Lemaitre, J., 2009. *Biomaterials*, 30(12): p. 2175-2179]. After adding the components listed in Table 3, in order, the volume of each solution was made up to 1 L with

distilled water. Both solutions are then sealed tightly and stored in two separate glass containers.

Prior to performing bioactivity analyses, both solutions were warmed to approximately 37 °C. An equal volume of each solution was then mixed. This mixture was then added to each of the samples to be studied and incubated for 6 h, 12 h and 24 h at 37 °C. The rationale for selecting these time points was that, for successful implantation, cells are expected to adhere to the surface and proliferate at the earliest. Therefore, a positive bioactive test with the least incubation period is crucial for a good implant surface [Chrzanowski, W., *et al.*, 2008. *Acta Biomaterialia*, 4(6): p. 1969-1984]. The samples were then washed thoroughly with distilled water and dried gently with compressed air. The growth of bone-like apatite layer on the surface was then studied using AFM.

Table 3 – Dual solution preparation of simulated body fluid

Formula	Solution A (g/L)	Solution B (g/L)
NaCl	6.129	6.129
NaHCO ₃	5.89	
Na ₂ HPO ₄ ·2H ₂ O	0.498	
CaCl ₂		0.54
HCl 1.00 M (mL/L)	0.934	0.934

Cell seeding

To obtain a preliminary estimate of the biocompatibility of the newly-modified PEEK surfaces, *in vitro* cell studies using the Human osteosarcoma cell line (MG-63) were employed. Cell seeding was preceded by sterilisation of all samples. The samples were first washed in 70% ethanol and allowed to remain in ethanol for 15 minutes, after which they were transferred to 24 well plates and washed with PBS twice. These samples were then seeded with 1×10^5 cells/well, following which they were incubated in a CO₂ incubator for 1 h at 37 °C. Media was then added to make the volume in each well up to 1 mL and incubated overnight at 37 °C.

Cell assay

To determine the metabolic activity of cells and their proliferation, an alamarBlue® assay was carried out on day 1, 4 and 7. 100 µL of the alamarBlue® dye (making up to 10% of media volume) was added to each well and incubated for 4 h at 37 °C. The fluorescence (at excitation 544 nm, emission 590 nm) was measured by transferring 200 µL aliquots from each well to a black plate and reading it using FLUOstar OPTIMA microplate reader.

Effect of temperature for high pressure carbon dioxide impregnation on surface topography of PEEK

The effect of temperature on the carbon dioxide impregnation of PEEK was studied by varying the temperature (40 °C, 60 °C and 80 °C), while keeping the pressure constant at 180 bar and employing a reaction time of 6 h. AFM measurements showed that maximum changes (in terms of increase or decrease in roughness) in surface topography were observed at 80 °C, 180 bar and for a reaction time of 6 h. AFM images of scan size of 20 µm have been presented here. From a visual observation of *Figure 9*, large grooves that are a result of polishing can be observed on naked polished PEEK, which then appeared to fade out on the 80 °C treated PEEK samples, *see, Figure 9(B)*.

When PEEK was subjected to high pressure carbon dioxide at 40 °C, unusual upright protrusions were seen which seemed to disappear on the 60 °C and 80 °C treated samples. A possible reason for this might have been that at a high pressure of 180 bar and temperature of 40 °C, in an attempt to form a smooth surface, the sharp ridge-like grooves and peaks on polished PEEK were forced to blend together, forming these protrusion-like structures. This may have been a precursor effect to the swelling phenomenon brought about by the carbon dioxide molecules. At higher temperatures, these structures probably began to melt away or deform to spread uniformly across the surface, thereby bringing about plasticisation of the surface. The sharp grooves and scratches, therefore appear to be less visible on the 80 °C treated sample in comparison to polished PEEK, *see, Figure 9(B)*.

This effect is further observed on the line profiles of the different surfaces presented in *Figures 10(A) and 10(B)*. The relatively sharp peaks and depressions observed on *Figure 10(A)* transformed to broader smoother profile lines on PEEK sample treated with carbon dioxide at 80 °C in *Figure 10(B)*. Moreover, another objective of optimising the conditions was to obtain nanoscale alterations on the surface of the sample. This was indicated by changes in nanoscale roughness with temperature treatment of PEEK (naked PEEK 17.87; 40 °C 15.73; 60 °C 10.86; 80 °C 20.12 nm).

In this study, 80 °C was chosen as the optimal temperature for further modification of PEEK, since it provided maximum evidence of plasticisation which was brought about by increased uniform surface smoothness accompanied by a uniform increase in nanoscale roughness. Uniform plasticisation is essential for the further successful impregnation of strontium-doped phosphate glass particles and nanodiamonds.

Strontium-doped phosphate glass particles impregnation of PEEK

The samples were placed in the Thar high pressure vessel. A thick paste of strontium-doped phosphate glass particles and 70% alcohol was pasted onto the polished surface of the samples. The impregnation was carried out as mentioned elsewhere at a temperature of 80 °C, a pressure of 180 bar and reaction time of 6 h. These samples are referred to as Sr-PEEK.

Following impregnation, the AFM images showed evident differences in surface topography in the form of appearance and distribution of small particle-like structures (~15-20 nm in height and ~0.5-1 µm in width) which were observed throughout the surface of PEEK. Moreover, the nanoscale roughness of the surface had decreased from 17.87 nm to 11 nm. This reduction in nanoscale roughness maybe affected by the increase in overall microscale roughness of the surface owing to the presence of these newly-formed structures. These changes in surface topography confirmed the successful impregnation of strontium-doped phosphate glass particles. Additionally, the samples showed an increase in weight of approximately ± 0.8 mg post-impregnation.

Incorporation of nanodiamond particles into PEEK

The samples were placed in the Thar high pressure vessel. A thick paste of nanodiamond particles and 70% alcohol was pasted onto the polished surface of the samples. The impregnation was carried out as mentioned elsewhere at a temperature of 80 °C, a pressure of 180 bar and reaction time of 6 h. Post surface modification, all samples were cleaned thoroughly in distilled water and then sonicated in alcohol for 15 minutes to removes any excess or unwanted particles on the surface. The changes in surface topography were then observed and studied using AFM.

Similar to strontium-doped glass particles impregnation, AFM scans carried out on nanodiamond impregnated PEEK samples showed the presence and uniform distribution of particle-like structures throughout the surface of PEEK as presented in *Figures 11(A) and 11(B)* and *Figure 12*. The maximum height of these structures were ~28 nm with a width of 0.5-1 µm (*see, Figure 13*). The roughness was evaluated to be 10 nm which was again less than that of naked polished PEEK. Besides these changes, a ± 3.0 mg increase in weight of the modified PEEK samples was recorded. These observed differences in surface topography and weight proved that nanodiamond particles were successfully impregnated

into the surface of PEEK. For convenience this sample will be referred to as ND-PEEK in the following sections.

Wettability

The wettability of the samples was determined by carrying out static contact angle measurements using a Kruss contact angle analyser (DSA-10 MK2). Here 3-4 separate droplets (approximately 0.5 μ L) of ultra pure water, diodomethane and formamide were placed on each sample, using automated syringe. The drop images were then captured immediately and their contact angles calculated. The surface free energy of the different samples was calculated using Extended Fowkes model and Owen's Wendt method via the Drop Shape Analysis software [Chrzanowski, W., *et al.*, 2010. *Journal of Biomedical Materials Research Part A*. 93A(4): p. 1596-1608; and Chrzanowski, W. *et al.*, 2012. *Journal of Materials Science: Materials in Medicine*, Volume 23, p. 2203-2215].

Generally, a larger contact angle is associated with increasing hydrophobicity of the surface. Hydrophobic surfaces generally also exhibit lower surface free energies [Spelt, D.L., 1992. *Modern Approaches to Wettability: Theory and Applications*. ed. Schrader, Plenum Press: New York; and Good, R.J., 1992. *Journal of Adhesion Science and Technology*, 6(12): p. 1269-1302]. It was observed that impregnation of PEEK with strontium-doped glass particles and nanodiamonds significantly increased the contact angle of the newly-modified surfaces (*Figure 14*). The water contact angle of ND-PEEK and Sr-PEEK was statistically greater than that of normal PEEK. Moreover the water contact angle of ND-PEEK exceeded 90° while that of Sr-PEEK remained relatively constant, which was indicative of a hydrophobic surface [Renate Forch, *et al.*, 2009. *Surface Design; Applications in Bioscience and Nanotechnology*, Wiley-VCH Verlag GmbH]. This rise in hydrophobicity of ND-PEEK maybe due to the presence of nanodiamonds, which are inherently hydrophobic in nature. *Table 4* provides a summary of contact angle, surface free energies and roughness of the different PEEK samples.

Table 4 – Wettability, surface free energy and roughness of samples

	Contact angle	Surface Free Energy (SFE)			Roughness
	Water (°)	SFE ^T (mN/m)	SFE ^D (mN/m)	SFE ^P (mN/m)	R _a ^{AFM} (nm)
PEEK	78.58	38.86	34.81	4.05	17.88
PEEK-CO ₂	70.8	41.15	34.22	6.93	19.64
PEEK-Sr	89.9	34.87	33.45	1.42	10.93
PEEK-ND	94.6	40.3	39.93	0.37	10

Interestingly, ND-PEEK showed an increase in surface free energy close to that of naked PEEK, which may explain the greater spreading of water droplets on its surface. However, the total surface energy was almost completely made up of the dispersion component (SFE^d), the polar component had little contribution to the total surface free energy (*Figure 15*). However, despite the hydrophobicity, ND-PEEK demonstrated heightened bioactivity and cellular activity potential.

Bioactivity – AFM measurements

The bone bonding ability of the newly-modified surfaces is evaluated by examining, using AFM, the ability of these surfaces to form a bone-like apatite layer. The degree to which the layer is formed in SBF determines the ability of the material to bond to a living bone [Kokubo, T. and H. Takadama, 2006. *Biomaterials*, 27(15): p. 2907-2915]. AFM images showed that there was good uniform layer growth for both nanodiamond and strontium glass-modified PEEK. This layer growth is confirmed by the appearance of small spherical precipitates that spread throughout the surface of the sample as seen in *Figures 16(A) and 16(B) and Figures 17(A) and 17(B)*. The size of these crystals appeared to increase with increasing incubation period in SBF. The appearance and morphology of these spherical precipitates were in agreement with findings in previous bioactivity studies [Yu, S., *et al.*, 2005. *Biomaterials*, 26(15): p. 2343-2352] and confirmed the presence of a hydroxyapatite layer. A relatively continuous layer growth that covers the entire surface indicates good bioactivity.

The samples were immersed in SBF for 6 h, 12 h and 24 h. However, only images after 6 h are presented here, since complete layer growth was observed within 6 h itself. No layer growth was observed for PEEK-CO₂ after 6 h, although more crystal precipitates were observed after 24 h. However contrary to what was expected, a layer of spherical precipitate was also observed on PEEK. This may, however, be due to remnant carbide particles that may have been present on the surface from polishing. However, naked PEEK was not directly responsible for growth of a bone-like apatite layer. Looking at the line profiles depicted in *Figures 18(A) and 18(B)*, which provide a representation of the cross-sectional characteristics of the apatite layer, it can be seen that the spherical crystals appearing on the naked PEEK are much smaller than those of Sr-PEEK and ND-PEEK.

Further, the line profile obtained for samples immersed in SBF for 24 h showed an increase in size of the spherical crystals. Larger magnification such as 10 μm and 20 μm

could not be retrieved for both ND-PEEK and SR-PEEK, since the sizes of the crystals went beyond the MFP capacity, meaning the height of these precipitates went beyond several micrometers in just under 24 h. This indicated a rapid increase in size and expansion of the hydroxyapatite layers. Naked PEEK, however, exhibited no increase in particle size, which affirms that naked PEEK was not directly responsible for the apparent bioactivity perceived in the 6 h images. Larger crystal size is associated with a surface having a higher bioactivity potential. It is important to note that the size of the spherical crystals detected on the ND-PEEK surface was larger than those observed for the other samples, suggesting that this new modification is capable of eliciting higher bioactivity *in vivo*.

Further, to confirm that ND-PEEK retained a higher bioactive potential than naked PEEK, new samples were taken and incubated in SBF for 1 h in order to observe which of the two samples formed an apatite layer more quickly. ND-PEEK, interestingly, showed a complete layer growth in just 1 h, suggesting that this material was more conducive to a quicker bioactive response *in vivo* (see, Figures 19(A) and 19(B); and Figures 20(A) and 20(B)).

In vitro cell metabolic activity and proliferation

The *in vitro* testing of the newly-modified surfaces was carried out using an alamarBlue® assay. Fluorescence is a direct measure of the number of viable cells in the media, thereby being an indicator of the cell proliferation performance. Fluorescence is measured using a microplate reader and is read at an excitation wavelength of 544 nm and emission wavelength of 590 nm. Naked PEEK was used as control sample. Since both Sr-PEEK and ND-PEEK showed outstanding bioactivity results by forming a bone-like apatite layer in less than 6 h, both these samples were expected to show competing high cellular activity for the *in vitro* studies.

As expected, the highest cellular activity was observed for ND-PEEK (Figure 21). However, contrary to what was expected, the metabolic activity for cells grown on Sr-PEEK showed lower cell proliferation than that of the naked PEEK sample. However an increase in cell activity was observed for all samples on day 4, which suggests that the new surface modifications did not elicit any form of toxicity to the cells; both modifications demonstrated advantageous biocompatibility properties.

From the above results, it can be concluded that although the surface of ND-PEEK was highly hydrophobic, as revealed by contact angle measurements, it elicited good

bioactivity by inducing apatite precipitation in less than 1 h. *In vitro* studies demonstrated that ND-PEEK elicits very good cell activity within 24 h, which suggests that the surface of ND-PEEK was conducive to instigating quick initial cell adhesion; this is generally required for good material integration with tissue. By day 4, ND-PEEK showed a further increase in cellular activity, which meant the surface provided an environment that was favourable for cell proliferation.

Although the surface free energy of PEEK was the highest, it can be concluded that this had very little effect, with the samples displaying favourable *in vitro* responses; dispersive forces expectably contributed to the majority of the surface free energy. The polar component was almost negligible in comparison. Accordingly, it may be concluded that the favourable *in vitro* response exhibited by PEEK could be a result of the specific chemical structure of nanodiamonds. Previous studies [Mochalin, V.N., *et al.*, 2012. *Nat Nano*, 7(1): p. 11-23; and Zhang, Q., *et al.*, 2012. *Biomaterials*, 33(20): p. 5067-5075] have shown that nanodiamonds support the growth and proliferation of murine osteoblasts and enhanced growth of an apatite-like layer when placed in SBF owing to favourable interactions with SBF ions.

Terpinen-4-ol functionalised PEEK surface for antimicrobial efficacy

This phase of the investigation related to fabricating an antimicrobial PEEK surface by the use of supercritical carbon dioxide – and to test the efficacy of the fabricated surface in the presence of live bacteria. Since it was established above that the solubility of the solute in supercritical CO₂ contributes to the impregnation efficiency, the fabrication method for this phase will depend on solubilising terpinen-4-ol in supercritical CO₂ and on the penetrability of supercritical CO₂ onto PEEK. The basis for the fabrication of an antimicrobial surface is for the supercritical CO₂ fluid to contain dissolved terpinen-4-ol; and for this to penetrate onto/into the surface of the PEEK, thus, embedding an amount of terpinen-4-ol into the polymer surface.

PEEK sample preparation

PEEK-Optima were obtained from *Invivio* in rod form, cut into 1.3 mm thick pieces using a Struers Accutom-50 diamond saw and polished using Rotopol 22 grinding equipment with 203 mm P#4000 silicon carbide paper. All samples were then washed with ethanol (60%, 70% and 90%) and pure water. The samples were prepared thereafter with

different treatments as shown in *Table 5*.

Table 5 – TTO sample abbreviated sample names

Abbreviated Name	Additional treatment
Naked PEEK	None
PEEK-CO ₂	Exposure to supercritical CO ₂
PEEK-son-ND	Impregnation with nanodiamond particles using supercritical CO ₂ at high pressure, high temperature and a 6 h duration
ND-PEEK	Impregnation with nanodiamond particles (sonicated for 1 h in 100% ethanol) using supercritical CO ₂ at high pressure, high temperature and a 6 h duration
PEEK-TTO-6h	Impregnation with terpinen-4-ol using supercritical CO ₂ at high temperature, high pressure and a 6 h duration
PEEK-TTO-24h	Impregnation with terpinen-4-ol using supercritical CO ₂ at low temperature, high pressure and a 24 h duration
PEEK-TTO-6h-Eth	Impregnation with terpinen-4-ol using supercritical CO ₂ at high temperature, high pressure and a 6 h duration with ethanol washing
PEEK-TTO-24h-Eth	Impregnation with terpinen-4-ol using supercritical CO ₂ at low temperature, high pressure and a 24 h duration with ethanol washing

5 *Supercritical carbon dioxide treatment and sample sterilisation of PEEK*

The inventive method of producing an antimicrobial surface was performed under the same conditions used to plasticise the surface of PEEK (180 bar, 80 °C and 6 h). However, this was accompanied with a slow depressurisation rate using 100 nm diameter capillary tubing. The quantity used for encapsulation and impregnation was approximately 10 50 µL terpinen-4-ol per PEEK sample. Temperature was varied between two limits (35 and 80 °C) and time was varied between two durations (6 and 24 h). The lower temperature was chosen as it is well above 31 °C, at which point CO₂ becomes supercritical. Sterilisation of PEEK-TTO samples was conducted during encapsulation and impregnation procedures by sterile handling. Other samples were sterilised using a standard protocol that utilises 70% 15 ethanol (v/v).

Particle size determination of sonicated nanodiamond particles

The mean particle size of nanodiamond particles was optimised by varying sonication durations with a Branson Sonifier 450 microtip, using output control of 30 and 20 keeping the duty cycle constant. Measurements were obtained from a Malvern Zetasizer Instrument and accompanying software.

Surface topography and roughness; physical and chemical characterisation

Modified sample surfaces were investigated under Atomic Force Microscopy (AFM) 25 to obtain imaging and topographic data. Mean roughness for the samples R_a were obtained

using the Gwyddion program.

NanoThermal Analysis using an Anasys Instrument was performed to measure the effects of using supercritical carbon dioxide to modify the surface of PEEK. The nano-TA probe was used to identify the points of local thermal property information such as the glass transition temperature T_g or the melting temperature T_m . Fourier Transform Infrared Spectroscopy (FTIR) fitted with Attenuated Total Reflectance (ATR) was used to determine the chemical composition of the altered surface of PEEK.

In vitro cellular response to different PEEK surfaces

Samples were sterilised using ethanol for 20 minutes and washed in PBS twice to remove residual ethanol. The cells used were MG63-human osteosarcoma cell. Seeding was done with 1×10^5 cells/cm² for each sample. This was incubated for 1 h at a temperature of 37 °C. Each well was topped up to 1 mL and allowed to incubate for 24 h at 37 °C. The alamarBlue® assay was carried out on time points (days) 1, 5, 8 and 14 day. 100 µL of the alamarBlue® dye was added into each well (to make 10% v/v) and incubation was performed for 4 h at 37 °C. 200 µL aliquots were transferred from each well to a black plate. Measurements were taken using FLUOstar OPTIMA microplate reader.

Apatite formation on different treated PEEK surfaces

The test was used to assess the viability of apatite growth on samples using a simulated body fluid solution (SBF). The preparation of SBF solution was performed as per Bohner and Lemaitre's study with the use of a mixture of two solutions A and B at room temperature and incubation of samples in solution was undertaken for 12 h [Bohner, M. and Lemaitre, J., 2009. *Biomaterials*, 30(12): p. 2175-2179].

Solution A was made up of 800 mL of distilled H₂O, 0.9 mL of 1 M HCl, 6.129 g of NaCl (Merck, Germany), 5.89 g of NaHCO₃ (Sigma-Aldrich, USA) and 0.394 g of Na₂HPO₄; Solution B was made up of 800 mL H₂O, 0.9 mL of 1 M HCl, 6.129 g of NaCl (Merck, Germany) and 0.54 g CaCl₂ (Merck, Germany). Both solutions were prepared separately in tight closed containers and stored in a dark room until needed. The solutions were mixed after bringing them to 37 °C and by simultaneously adding equal volumes over time. Samples were immersed in SBF solution with active side facing downwards in a conical centrifuge tube. Incubation of samples was performed at 37 °C. Samples were taken out for analysis under Atomic Force Microscope and returned at nominated time points.

Inhibition zones of different treated surfaces

Mueller-Hinton plates were prepared by mixing approximately 22 g Mueller Hinton powder to 1 L of deionised water to make 1 L solution. Agar powder was added to the mixture and mixed using magnetic stirrer while adjusting to pH 7.2 using a pH probe. Another two 5 mL Mueller Hinton broths were separated in sterile McCartney bottles for swabbing confluent lawn. The Mueller Hinton agar broths were autoclaved and left to cool to 37 °C whereupon they were poured into agar plates and left to solidify. Subcultures were prepared using strains of *S. aureus* and *P. aeruginosa*. The agar plates were swabbed to make confluent lawn with one colony per 5 mL in the McCartney bottles and samples were placed facing down onto the agar. Samples were kept in 37 °C room for 18 h. Zones were measured using a ruler.

Bacterial adhesion onto different treated surfaces

Subcultures were prepared using *S. aureus* and *P. aeruginosa* in 80 mL Mueller Hinton broth and incubated at 37 °C for 18 h. Inoculation of one colony per 5 mL was performed and the broth was retained on a shaker at 37 °C during a 2 h log phase. 40 mL broth was poured into each petri dish and sterile samples were added. Tests were accomplished in duplicates.

Samples were then removed at time points 10 minutes, 1 h and 24 h and placed into 24 well plates for fixing for 20 minutes and washed twice with phosphate buffer solution. Samples were stored in a cool room after fixing and washing while left saturated in PBS until imaging. Scanning electronic microscopy (SEM) was used to investigate the attachment of organisms on the surface.

The fabrication of PEEK-son-ND was achieved by the addition of 1 h sonication (30 output control, constant duty set point) at the previously-determined parameters of 80 °C, 180 bar and 6 h. Although impregnation managed to make no significant changes to the surface mean roughness and wettability, results for cell viability and apatite formation tests suggest that the fabricated PEEK-son-ND possessed surface properties that were conducive for cell and apatite modulation. It was concluded that the presence of nanodiamonds promoted the *in vitro* modulation of apatite growth without a significant change in the morphology and chemical characteristics of the fabricated PEEK-son-ND surface. Two sample types were fabricated using the previously-determined parameters of 6 h at 80 °C for

optimum impregnation; and 24 h treatment at 35 °C. Pressure was maintained throughout the two variations at 180 bar. A second variation, of temperature 35 °C, was chosen to be slightly above 31 °C (where carbon dioxide reaches supercritical state). Wettability, thermal mechanical and FTIR-ATR results determined the key differences between the two methods of fabrication and thus, opted for the method that produced the surface most suitable for functionalisation. Zone inhibition and bacterial adhesion tests confirmed the efficacy of fabricated antimicrobial PEEK surface.

Terpinen-4-ol solubility in supercritical CO₂

One of the many known uses of supercritical CO₂ is in extraction of essential oils from leaves and plants; components of Tea Tree Oil were successfully extracted by using supercritical CO₂. The loading of terpinen-4-ol onto individual PEEK surfaces remained constant at approximately 50 µL/sample. The results suggested that the solubility limit in supercritical CO₂ of terpinen-4-ol was exceeded when 300 µL of terpinen-4-ol was added. This essentially means that supercritical CO₂ was only successfully able to solubilise an amount ranging between 0 to 300 µL before it became saturated. Thus, anything above the threshold was not solubilised and remained on the surface of the PEEK, thereby producing the visual coating observed on the samples.

Effect of supercritical CO₂ method on surface chemical composition

Fourier Transform Infrared Spectroscopy (FTIR) fitted with Attenuated Total Reflectance (ATR) was used to determine the presence of terpinen-4-ol on the surface of fabricated PEEK-TTO. The first FTIR-ATR spectrograph revealed the presence of characteristic peaks of terpinen-4-ol present on the surface of PEEK-TTO-6h, as shown in *Figure 22(B)*. This information was compared to the spectrograph produced from approximately three drops of pure terpinen-4-ol solution, as shown in *Figure 22(A)*. Terpinen-4-ol is predominantly composed of aromatic C-C, C=C, -H and R-OH bonds. The spectra shown in *Figure 22(A)* and *Figure 22(B)* revealed similar peaks on both measurements. The second observation was that a few characteristic peaks in PEEK-TTO-6h varied in intensity from pure terpinen-4-ol which indicated a varied concentration of corresponding chemical bonds present on PEEK-TTO-6h. Interpretation of the similarities revealed in the data lead to the conclusion that a substantial amount of hydrocarbon and aromatics in terpinen-4-ol could be detected on the PEEK surface as a result of supercritical

CO₂ treatment. Comparison of the spectrographs also revealed a large difference in the characteristic peak of R-OH (alcohol) between the two, indicating that there is not as much of this chemical group detected on the surface of PEEK-TTO-6h. Analysis of the FTIR-ATR outputs convincingly validated the efficacy of supercritical fluid on the fabrication of the PEEK-TTO-6h surface. The decreased detection of alcohol was attributed to evaporation during sample preparation or dissolution in CO₂ during system depressurisation. An additional hypothesis that was made was that terpinen-4-ol, which has a flashpoint of 50 °C, encouraged the loss of some alcohol during depressurisation while temperature was slowly brought down from 80 °C to ambient. However, FTIR-ATR results were non-quantitative and should not be used to evaluate the concentration of the terpinen-4-ol. Nevertheless, the detection of characteristic terpinen-4-ol peaks on the sample clearly validated the efficacy of using the supercritical CO₂ method in the fabrication of PEEK-TTO.

Effect of supercritical CO₂ impregnation on PEEK topography and morphology

The effects of supercritical CO₂ treatment on the surface of PEEK-TTO-6h (6 h, 180 bar and 80 °C) was assessed using atomic force microscopy (AFM). Results revealed that PEEK-TTO-6h possessed a reduced roughness average parameter R_a of 10 nm. Meanwhile, AFM imaging showed traces of scattered matter in some regions of the PEEK-TTO-6h surface. The traces were thought to be the layer of terpinen-4-ol sitting directly on the surface of the sample. In *Figure 23*, puddle-like features manifest on the edge, amounting to a rough approximated maximum height of 40 nm and an approximated coverage of 0.5 μm².

Results indicate a nanoscale smoothening effect with supercritical CO₂ treatment in the fabrication of PEEK-TTO-6h (naked PEEK 18.2; PEEK-TTO-6h 10.0 nm). Significantly, adding 50 μL terpinen-4-ol would result in excess compound detectable by AFM on the surface of the sample. Visual detection of the terpinen-4-ol layer confirmed antimicrobial compound presence on the surface and further inspired the idea of preserving the thin layer of terpinen-4-ol to inhibit bacterial attachment during the initial stages of implant surgery.

Effects of temperature and time variation on PEEK-TTO (80 °C, 6 h vs. 35 °C, 24 h)

The studies were targeted to determine which method (80 °C and 6 h vs. 35 °C and 24 h) fabricated PEEK with a higher inclination to retain more terpinen-4-ol after ethanol washing. After supercritical CO₂ treatment, samples PEEK-TTO-6h, PEEK (samples treated

with supercritical CO₂ for 6 h at 80 °C) and PEEK-TTO-24h (samples treated with supercritical CO₂ for 24 h at 35 °C) were washed with 70% ethanol (v/v) as terpinen-4-ol was found to be highly soluble in ethanol. Samples pre- and post-washing were compared and the results show the relative state of each sample.

5

Surface chemical composition

Ethanol washing easily removed most of the terpinen-4-ol previously present on the surface of PEEK-TTO-6h and PEEK-TTO-24h. FTIR-ATR was not able to detect anything on the fabricated samples due to the small remnant quantity of terpinen-4-ol left after ethanol washing. The results of this test indicate that both methods achieved the same strength of terpinen-4-ol coating, on the basis that the same effect after washing for both samples was achieved.

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Wettability

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Results from the sessile droplet technique were aimed to show the degree of hydrophobicity and surface energy change between naked PEEK and fabricated samples PEEK-TTO-6h and PEEK-TTO-24h – and also the difference after the samples were washed in ethanol. The results were presented in *Figure 24* and *Figure 25*, respectively, by way of the samples' water contact angle as a measure of hydrophobic character – and their final surface free energies, as well as their polar and dispersive components pre- and post-ethanol washing. The water contact angle test results revealed that the hydrophobic state of the modified surface after ethanol washing for both PEEK-TTO-24h and PEEK-TTO-6h were statistically the same as found in the pre-washing state (*see, Figure 25*). However, there was a noticeable drop in hydrophobic character for PEEK-TTO-24h after supercritical CO₂ treatment, which remained constant after ethanol washing.

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The main observation made from the surface free energy calculation results was that PEEK-TTO-6h resulted in a large increase in final surface free energy after supercritical CO₂ treatment. The overall observations for all other samples were that they were statistically not different from naked PEEK. After ethanol washing, both PEEK-TTO-6h and PEEK-TTO-24h resulted in total surface energy differences of no statistical significance ($p < 0.05$). For all samples, the dominant component was observed to be the dispersive component, indicating no major change to the hydrophobic nature of any sample.

30

Effect of supercritical CO₂ method on surface thermal mechanical properties

Nano-TA results revealed the comparisons between thermal mechanical property profiles across 25 localised sample points on a 10 x 10 µm surface of the samples naked PEEK, PEEK-TTO-6h and PEEK-TTO-24h.

5 The data depicted in *Figure 26* reveal that overall, sampling curves were uniform throughout the three samples. This in turn indicates that the method of fabrication did not affect the structural integrity of any sample under comparison. None of the methods produced samples with a significant inclination to retain terpinen-4-ol on the modified surface after ethanol washing. However, for the purposes of the antibacterial and cell
10 viability studies, the fabrication with a 6 h time and 80 °C temperature (PEEK-TTO-6h) was chosen.

Effect of PEEK-TTO-6h on cell viability

To assess the effect of the fabricated PEEK-TTO-6h (PEEK treated for 6 h, 80 °C
15 and 180 bar) on cellular activity, the count of cells from time point day 1 to day 8 were recorded and presented in *Figure 27*. The results revealed that PEEK-TTO-6h showed signs of toxicity to cells from days 1 to 8. The count of cells exposed to PEEK-TTO-6h was much lower than that of naked PEEK over all days. A cell count drop in control media from day 5 to day 8 was recorded, while succeeding day measurements in cell media showed cellular
20 activity had reached a plateau. Interestingly, cellular activity continued to increase from day 5 to day 8 on naked PEEK and PEEK-TTO-6h despite the observed drop in cell media.

Formation of zones of inhibition around different treated surfaces

This study was performed to determine whether a ring of decreased bacterial density
25 around the edge of the fabricated PEEK-TTO-6h can be produced. The results of this study showed that no zones of inhibition were produced around all four samples: PLA with silver, PEEK-CO₂, naked PEEK and PEEK-TTO. There was visual confirmation of confluent bacterial lawn formed over an 18 h incubation period, ruling out the possibility of faulty plates or no bacterial growth. This outcome revealed that none of the samples eluted a
30 diffusible medium of antimicrobial compounds from their surfaces, including PLA with silver, which had demonstrated potential antimicrobial diffusing mechanism in previous studies.

Bacterial adhesion onto different treated surfaces

This study investigated the efficacy of the fabricated PEEK-TTO-6h on impairing bacterial attachment and adhesion. The bacterial adhesion tests were performed for incubation time points of 10 minutes, 1 h and 24 h for common hospital-acquired bacteria *S. aureus* and *P. aeruginosa*.

The first images obtained from atomic force microscopy (AFM) gave no visual confirmation of bacteria present on the surfaces of naked PEEK and PEEK-TTO-6h for the time point 10 minutes, tested with *S. aureus*. There was no detection of bacterium-like structures present on the sample surface. The outcome suggested that AFM imaging was not suitable for short-time bacterial attachment detection and that scanning electron microscopy (SEM) was more appropriate for confirming the presence of bacteria on the surface; it is able to show much broader imagery of the sample surface. SEM imaging results for 10 minute time point naked PEEK and PEEK-TTO-6h samples were obtained and shown in *Figure 28(A)* and *28(B)*; and *Figures 29(A)* and *29(B)* for samples tested with *P. aeruginosa* and *S. aureus* respectively. It was observed that groups of *P. aeruginosa*, colonies could be visually spotted in various regions on the surface of naked PEEK while PEEK-TTO-6h was noticeably sparse in bacterial density on the surface. Another observation was that colonies of *P. aeruginosa* present on the surface of PEEK-TTO-6h appeared not to be structurally intact (see, *Figure 28(B)*, yellow arrows). Good representative images of the collective observations made are shown in *Figures 28(A)* and *28(B)*.

Meanwhile, groups of what appeared to be *S. aureus* colonies were spotted in some regions on the surface of naked PEEK. It was observed that PEEK-TTO-6h had no obvious accumulation of the clusters of bacteria present on the naked PEEK. Visual investigations made of PEEK-TTO-6h suggested there was little bacterial attachment on the surface in that short 10 minute time point. *Figures 29(A)* and *29(B)* show representative views that best resembles overall conditions of sample surfaces.

Conclusions drawn from experimental methods and results

Changes in surface topography in terms of a decrease in nanoscale roughness and the appearance of a uniformly smooth surface in comparison to naked polished PEEK affirmed this result. These conditions were further successfully used to modify the surface of PEEK with nanodiamonds (*i.e.*, ND-PEEK) and strontium-doped phosphate glass particles (Sr-PEEK). Analysis of the surface topography revealed the appearance of small particle-like

structures on the surface. Additionally, there were slight variations in R_a between PEEK ($R_a \sim 17.88$ nm) and the newly-modified surfaces of ND-PEEK ($R_a \sim 10.93$) and Sr-PEEK ($R_a \sim 10$). However, no evidence of a positive influence of these nanoscale variations on either bioactivity or cell behaviour were observed, since both PEEK and ND-PEEK evoked more favourable cell responses in comparison to the other samples. Apart from surface roughness and topography, the surface free energy is another factor that determines the bioactivity of the material; this was evaluated by determining the contact angle measurements and surface free energy.

The water contact angle was measured to be highest for ND-PEEK (94°), followed by Sr-PEEK (89.9°) and lowest for CO₂-PEEK (70.8°), with naked PEEK (78.58°) sitting between the reported extremes. The high contact angles value of ND-PEEK and Sr-PEEK were indicative of hydrophobic tendencies. However, previous studies [Kokubo, T., *et al.*, 1996. *Journal of the American Ceramic Society*, 79(4): p. 1127-1129] have suggested that hydrophilic rather than hydrophobic surfaces are better inducers of bioactivity and cell responses. Interestingly, ND-PEEK elicited the most favourable and quickest bioactivity and cell behaviour response. This may be attributed to the higher surface free energy (40.3 mN/m) exhibited by ND-PEEK surfaces. However, despite the high surface free energy exhibited by CO₂-PEEK (41.15 mN/m), its influence on bioactivity and cell behaviour were relatively poor; indeed, dispersive forces contributed to much of the recorded surface free energy. Therefore, besides surface free energy and topography, there may have been other factors that were responsible for the enhanced bioactivity of ND-PEEK.

The ability of the new surface modifications to induce bioactivity *in vivo* was investigated by analysing the formation of a bone-like apatite layer on the sample surface using AFM. A continuous layer of hydroxyapatite was observed within 6 h on both Sr-PEEK and ND-PEEK. These appeared as small spherical crystal precipitates which spread across the surface of the sample. The morphology and appearance and growth pattern of these precipitates were in agreement with previous studies [Kokubo, T. and H. Takadama, 2006. *Biomaterials*, 27(15): p. 2907-2915; and Yu, S., *et al.*, 2005. *Biomaterials*, 26(15): p. 2343-2352], hence affirming the build-up of bone-like hydroxyapatite layer on the sample surface. The formation of this apatite layer in less than 6 h verified the enhanced bioactivity potential of both Sr-PEEK and ND-PEEK. Immersion of the samples in SBF for 24 h resulted in further film growth in other samples; no progressive growth was observed on PEEK. However, it is important to note that the surface of ND-PEEK seemed to evoke the

fastest (less than 1 h) precipitation of bone-like apatite and also showed greater growth rate of layer in terms of size of the particles and expansion over the surface. Based on the above observations ND-PEEK and Sr-PEEK were expected to elicit higher growth and cell proliferation in *in vitro* studies.

5 A preliminary *in vitro* cell study confirmed that both surface modifications, *i.e.*, Sr-PEEK and ND-PEEK supported cell growth and proliferation. However analysis of cell behaviour demonstrated that the most favourable cell response was observed for ND-PEEK.

 The results of cell behaviour were not in complete agreement with the bioactivity evaluation especially for Sr-PEEK. Significantly higher cell proliferation, in comparison to
10 naked PEEK, was expected for both Sr-PEEK and ND-PEEK.

 There is a possibility that the bioactivity and cell proliferation potential of the new surface modifications were considerably reduced due to being subjected to a relatively long period of sonification (15 minutes), as noticeable differences in particle distribution were found on Sr-PEEK and ND-PEEK images obtained before and after sonication was carried
15 out; some of the impregnated nanoparticles may have been lost in the process of sonication. Most biomaterials are sonicated for around 5 minutes, if the objective is to remove excess loose particles instead of more severe contaminants.

 The sonicated samples elicited a good bioactive response, which meant that even small amounts of these particles were capable of enhancing cell responses. It can be
20 concluded the results obtained for ND-PEEK were in agreement with the established hypothesis that the significantly enhanced bioactivity of ND-PEEK and the associated cellular activity makes it a suitable biomaterial for bone tissue engineering applications. Sr-PEEK, on the other hand, would require further preliminary testing to confirm its suitability as a potential bone tissue engineering biomaterial.

25 The overall outcome of this investigation confirmed the potential of using supercritical CO₂ surface treatments as viable method to improve the functional properties PEEK implants on the surface. Successful functionalisation of PEEK surfaces was achieved as the final outcome of this investigation with the incorporation of nanodiamond and terpinen-4-ol with optimum parameters of 80 °C, 6 h and 180 bar. Cell viability studies
30 indicate the toxic threshold for human cell line MG-63 was encountered at 50 µL terpinen-4-ol per active sample.

 It was crucial to optimise parameters for the deposition of terpinen-4-ol on the surface of PEEK. Thus, two fabrication methods applying temperature and time variations

were employed. Wettability, thermal mechanical and FTIR-ATR measurements produced similar results between the two methods of fabrication. This indicated that neither of the two fabrication methods achieved a higher deposition of terpinen-4-ol on the sample surface and thus, supercritical carbon dioxide treatment for the fabrication of antimicrobial PEEK surfaces was performed at the chosen parameters of 6 h, 80 °C and 180 bar. Characteristic peaks found on the FTIR spectra which indicated the detection of terpinen-4-ol on the surface of PEEK confirmed the efficacy of fabrication method. Zone of inhibition and bacterial adhesion tests two strains of bacteria, *P. aeruginosa* and *S. aureus*, resulted in the high efficacy of fabricated antimicrobial PEEK surface to inhibit bacterial adhesion, while zones of inhibition were not developed for any of the samples.

The fabrication of modified PEEK surfaces for enhanced functional implants through the use of supercritical CO₂ was successful as a preliminary study and showed a good potential for subsequent developments. The previously-determined parameters of 80 °C, 6 h and 180 bar proved optimal for the impregnation of nanodiamond particles and the fabrication of antimicrobial PEEK surfaces.

Fabrication of PEEK-son-ND was successful and validated using AFM phase imaging. Despite having no significant changes from impregnation, the presence of diamonds on the surface of PEEK-son-ND successfully induced increased cellular adhesive activity and proliferation. Cell viability tests indicated positive results for PEEK-son-ND with consistent increased cellular activity observed from day 1 to day 5. No toxic effects eluted from the PEEK-son-ND surface could be detected which confirms the viability of this study *in vivo*.

The second phase of the present invention focused on the development of antimicrobial PEEK surfaces. Results from the optimisation of the fabrication method and subsequent ethanol washing confirmed that the effects of temperature and time variation did not improve the incorporation of terpinen-4-ol onto the surface of PEEK. The two methods varied between 35 and 80 °C and 24 and 6 h, respectively. Results from FTIR-ATR characterisations, wettability measurements, SFE calculations and thermal-mechanical property measurements have collectively suggested that none of the two methods had a leverage of increased terpinen-4-ol deposition on the PEEK surface. Nano-thermal mechanical analysis revealed there was no significant changes made to the fabricated sample surface. Results indicate no significant differences between samples fabricated via two methods and thus, the preferred fabrication method employed conditions of 6 h, 80 °C and

180 bar for ease of application.

The fabrication of PEEK-TTO-6h (PEEK supercritically treated at 80 °C, 180 bar and for 6 h) was successful by detection of characteristic alcohol, aromatic and hydrocarbon bonds found on the IR spectra of the fabricated PEEK-TTO-6h by surface chemical
5 characterisations performed on FTIR-ATR. Supercritical CO₂ treatment affected the surface average roughness profile, as reflected by the decrease of the roughness average parameter R_a to 10 nm from 18 nm.

Cell viability studies showed obvious toxic effect of PEEK-TTO-6h samples to 1 x 10⁵ cells/cm² sample. Cellular activity was found to drop with exposure to concentrations of
10 approximately 50 µL terpinen-4-ol per active sample surface area. The cytotoxic activity of terpinen-4-ol towards human cell lines MG-63 is most likely concentration-dependent. One potential future line of investigation as to the feasibility of this invention in biomedical implants would be to determine the percentage of terpinen-4-ol versus cell survival in count.

Inhibition zone studies revealed that PEEK-TTO-6h was not capable of producing a
15 maximum inhibitory zone due to the lack of antibacterial compound deposit on the surface. Therefore, an alternative study to determine bacterial sensitivities to the fabricated sample may be appropriate.

The capacity of PEEK-TTO-6h to inhibit bacterial attachment and adhesion of two strains of bacteria, *P. aeruginosa* and *S. aureus*, was successfully demonstrated through
20 SEM confirmations of dying bacteria on the fabricated antimicrobial surface at 37 °C incubation time point of 10 minutes. Similar or improved results can be extrapolated for the 1 h and 24 h incubation samples. In any case, the short time-point results provide a solid preliminary framework for future studies.

Although the invention has been described with reference to specific examples it will
25 be appreciated by those skilled in the art that the invention may be embodied in many other forms.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method for enhancing the biomedical profile of an implantable material, the method comprising the steps of:
 - 5 selecting the implantable material;
 - subjecting the implantable material to a first treatment step comprising exposure to supercritical carbon dioxide, at a first predetermined temperature, under a first predetermined pressure, over a first predetermined time, thereby to plasticise at least a portion of the surface of the implantable material;
 - 10 subjecting the plasticised surface of the implantable material to a second treatment step, performed concurrently with or subsequent to the first treatment step, the second treatment step comprising exposure to an additive material in a medium of supercritical carbon dioxide, at a **second** predetermined temperature, under a second predetermined pressure, over a second predetermined time, thereby to
 - 15 impregnate at least some of the additive material into at least a portion of the plasticised surface of the implantable material, thereby to provide a composite implantable material.
2. A method according to claim 1, wherein the implantable material comprises
20 polyether ether ketone (PEEK).
3. A method according to claim 1, wherein the implantable material is comprised of, or consists essentially of polyether ether ketone (PEEK).
- 25 4. A method according to any one of the preceding claims, further comprising performing a post-treatment washing step, thereby to remove at least some non-embedded, or non-chemically-associated particles of the additive material (*i.e.*, unreacted additive material).
- 30 5. A method according to any one of the preceding claims, wherein the biomedical profile of the implantable material is enhanced with respect to the biocompatibility of the composite material; or the resistance of the composite material to bacteria.

- 5
6. A method according to any one of the preceding claims, wherein the first predetermined temperature is between about 31 and about 100 °C, the first predetermined pressure is between about 73 and about 200 bar and the first predetermined time is between about 0.2 hours and about 48 hours.
7. A method according to claim 6, wherein the first predetermined time is between about 0.2 hours and about 24 hours.
- 10
8. A method according to claim 6 or claim 7, wherein the first predetermined temperature is about 80 °C, the first predetermined pressure is about 180 bar and the first predetermined time is about 6 hours.
- 15
9. A method according to any one of the preceding claims, wherein, if the second treatment step is not performed concurrently with the first treatment step, the second predetermined temperature is between about 31 and about 100 °C, the second predetermined pressure is between about 73 and about 200 bar and the second predetermined time is between about 0.2 hours and about 48 hours.
- 20
10. A method according to claim 9, wherein the first predetermined time is between about 0.2 hours and about 24 hours.
- 25
11. A method according to claim 9 or claim 10, wherein the second predetermined temperature is about 80 °C, the second predetermined pressure is about 180 bar and the second predetermined time is about 6 hours.
12. A method according to any one of the preceding claims, further comprising the step of polishing the surface of the implantable material prior to undertaking the first treatment step.
- 30
13. A method according to any one of the preceding claims, wherein the surface modified implantable material exhibits bulk thermal and mechanical properties that are not reduced to a statistically-significant degree with respect to those of the untreated implantable material.

14. A method according to claim 5, wherein the biomedical profile of the implantable material is altered by enhancing the biocompatibility of the material.
- 5 15. A method according to claim 14, wherein the additive material is a plurality of nanodiamonds.
- 10 16. A method according to claim 14 or claim 15, wherein during the second treatment step, the nanodiamonds are provided as a suspension in an alcoholic carrier prior to being subjected to the supercritical carbon dioxide.
17. A method according to claim 16, wherein the alcohol comprises ethanol.
- 15 18. A method according to claim 16, wherein the step of providing the nanodiamonds in a suspension of ethanol comprises pasting the suspended nanodiamond particles onto the surface of the implantable material prior to subjecting the reactant materials to the second treatment step comprising supercritical carbon dioxide impregnation.
- 20 19. A method according to claim 17 or claim 18, wherein the suspension of nanodiamonds in ethanol has a solids content of between about 5 mg/mL and about 100 mg/mL.
- 25 20. A method according to claim 19, wherein the suspension of nanodiamonds in ethanol has a solids content of about 10 mg/mL.
21. A method according to any one of claims 17 to 20, further comprising a sonication step, thereby to reduce any agglomeration of the nanodiamonds suspended in the ethanol.
- 30 22. A method according to claim 20, wherein the sonication step is defined by parameters comprising a period of about 2 h; an output control setting of about 20 kHz, 500W; and a constant duty cycle.

23. A method according to any one of claims 15 to 22, wherein the biocompatibility of the material is enhanced by the impregnation of nanodiamonds upon at least a portion of the surface of the implantable material, the impregnated nanodiamonds providing a platform for the fusion of a hydroxyapatite (HA) layer with the composite surface, the HA layer subsequently providing a platform to enhance bone mineralisation.
24. A method according to claim 23, wherein the HA layer also serves to enhance osteoblast adhesion and proliferation.
25. A method according to any one of claims 15 to 24, wherein the surface roughness average parameter R_a of the untreated implantable material reduces after the first treatment step and reduces further after the second treatment step.
26. A method according to claim 25, wherein the surface roughness average parameter R_a for nanodiamond-impregnated PEEK is in the desired range to stimulate cell responses.
27. A method according to claim 25 or claim 26, wherein the surface roughness average parameter R_a for nanodiamond-impregnated PEEK, having undergone about 2 h of post-treatment sonication is between about 10 and about 50 nm, preferably between about 10 and about 20 nm.
28. A method according to any one of claims 25 to 27, wherein the surface roughness average parameter R_a for nanodiamond-impregnated PEEK, having undergone about 15 minutes of post-treatment sonication is about 14.2 nm.
29. A method according to any one of claims 15 to 28, wherein the post-treatment washing step employs distilled water, followed by sonication in ethanol for about 15 minutes, thereby to remove any excess or non-impregnated (*i.e.*, unreacted) nanodiamond particles from the surface of the composite material.
30. A method according to any one of claims 15 to 29, wherein the nanodiamond-

impregnated PEEK samples show a relatively uniform distribution of particle-like structures upon the surface of the treated PEEK.

- 5 31. A method according to claim 30, wherein the individual particle-like structures have an average height of between about 10 and about 40 nm..
- 10 32. A method according to any one of claims 15 to 31, wherein the nanodiamond-impregnated PEEK exhibits an increased contact angle in wettability tests relative to untreated PEEK, said increased contact angle indicative of the increased hydrophobicity of the composite surface.
33. An implantable material having a biomedical profile enhanced by a method as defined according to any one of claims 1 to 32.
- 15 34. An implantable material according to claim 33, wherein the impregnation of nanodiamonds upon the surface of the implantable material does not significantly alter the bulk thermal mechanical properties of the implant for load-bearing applications, thereby ensuring the required functional matching to hard bodily tissue.
- 20 35. An implantable material according to claim 33 or claim 34, wherein the modified implantable material couples the favourable bio-response enhancing profile of nanodiamonds with the advantageous mechanical properties of PEEK, thereby to render the composite material amenable for use as a bone biomaterial.
- 25 36. A method according to claim 5, wherein the biomedical profile of the implantable material is altered by enhancing the resistance of the material to bacteria.
37. A method according to claim 36, wherein the additive material is terpinen-4-ol.
- 30 38. A method according to claim 36 or claim 37, wherein as an optional precursor step, the terpinen-4-ol is extracted from Tea Tree Oil using supercritical carbon dioxide.
39. A method according to claim 36 or claim 37, wherein the terpinen-4-ol is sourced

commercially.

- 5 40. A method according to any one of claims 36 to 39, wherein the step of providing the terpinen-4-ol comprises pasting about 50 μ L of terpinen-4-ol onto the surface of the implantable material prior to subjecting the reactant materials to the second treatment step comprising supercritical carbon dioxide impregnation.
- 10 41. A method according to any one of claims 36 to 40, wherein the washing step comprises ethanol, thereby to wash away terpinen-4-ol that is not chemically associated with the surface of the composite material (*i.e.*, unreacted) following the second treatment step with supercritical carbon dioxide.
- 15 42. A method according to any one of claims 36 to 41, wherein the composite terpinen-4-ol/ CO_2 -impregnated PEEK exhibits greater hydrophobicity than does untreated PEEK.
- 20 43. A method according to any one of claims 36 to 42, further comprising the step of employing a slow depressurisation rate using 100 nm diameter capillary tubing following the first and/or second treatment steps.
44. A method according to any one of claims 36 to 43, wherein the composite material of terpinen-4-ol-impregnated PEEK shows resistance to *S. aureus* and *P. aeruginosa* for incubation time points of 10 minutes, 1 h and 24 h.
- 25 45. An implantable material having a biomedical profile enhanced by a method as defined according to any one of claims 1 to 13, or any one of claims 36 to 44.
- 30 46. An implantable material according to claim 45, wherein the impregnation of nanodiamonds upon the surface of the implantable material does not significantly alter the bulk thermal mechanical properties of the implant for load-bearing applications, thereby ensuring the required functional matching to hard bodily tissue.
47. An implantable material according to claim 45 or claim 46, wherein the modified

implantable material couples the favourable antibacterial-enhancing profile of terpinen-4-ol with the advantageous mechanical properties of PEEK, thereby to render the composite material amenable for use as an implantable material that may inhibit bacterial attachment during the initial stages of implant surgery.

5

48. An implantable composite material having nanodiamonds impregnated within at least a portion of the surface area of the implantable material, the nanodiamond-impregnated implantable material thereby having an enhanced bone bonding ability (integration with bone) relative to an equivalent material without the impregnated nanodiamonds.

10

49. An implantable composite material having terpinen-4-ol impregnated within at least a portion of the surface area of the implantable material, the terpinen-4-ol-impregnated implantable material thereby having an enhanced antibacterial profile relative to an equivalent material without the impregnated terpinen-4-ol.

15

50. A method of performing orthopaedic surgery, said method comprising implanting at a surgical site a material as defined according to any one of claims 33 to 35, or claim 48.

20

51. A method of performing orthopaedic surgery, said method comprising implanting at a surgical site a material as defined according to any one of claims 45 to 47, or claim 49.

25

52. Use of an implantable composite material as defined according to any one of claims 33 to 35, or claim 48, in orthopaedic surgery.

53. Use of an implantable composite material as defined according to any one of claims 45 to 47, or claim 49, in orthopaedic surgery.

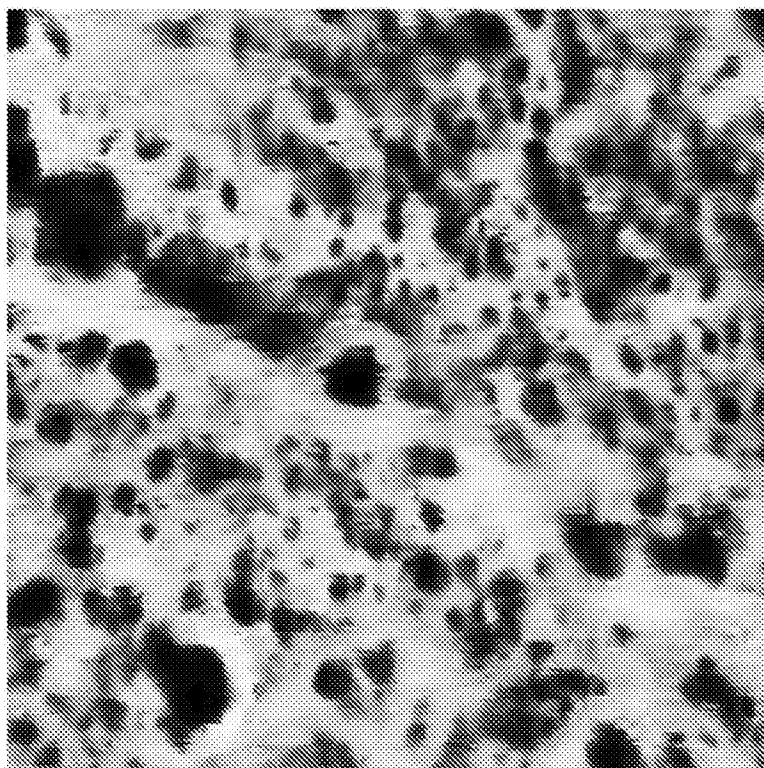


FIG. 1

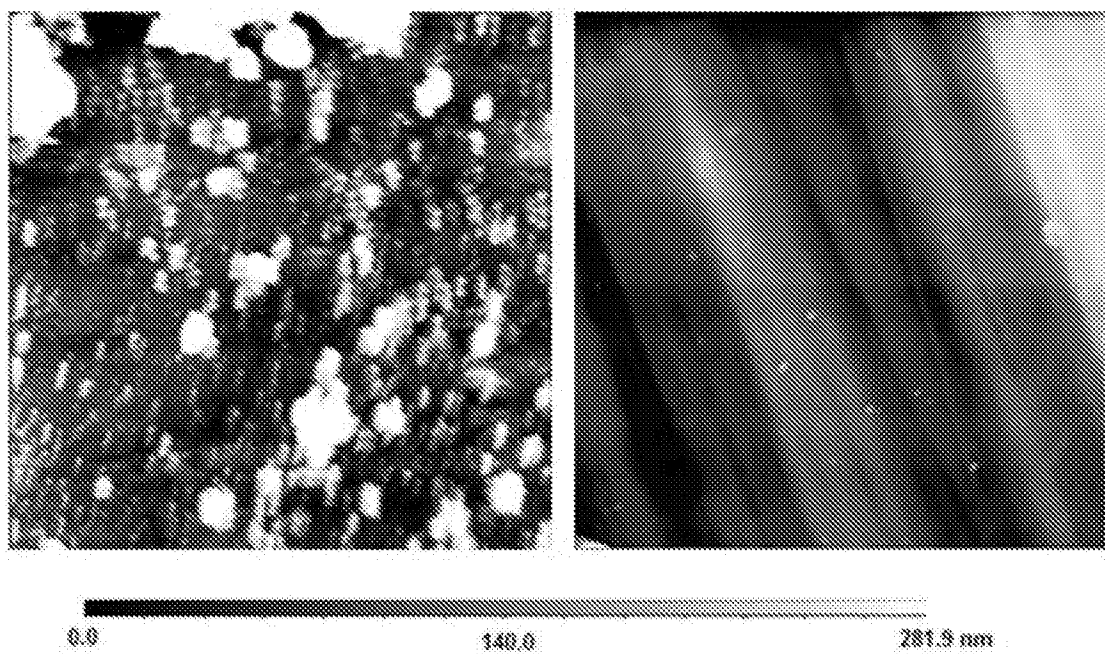


FIG. 2(A)

FIG. 2(B)

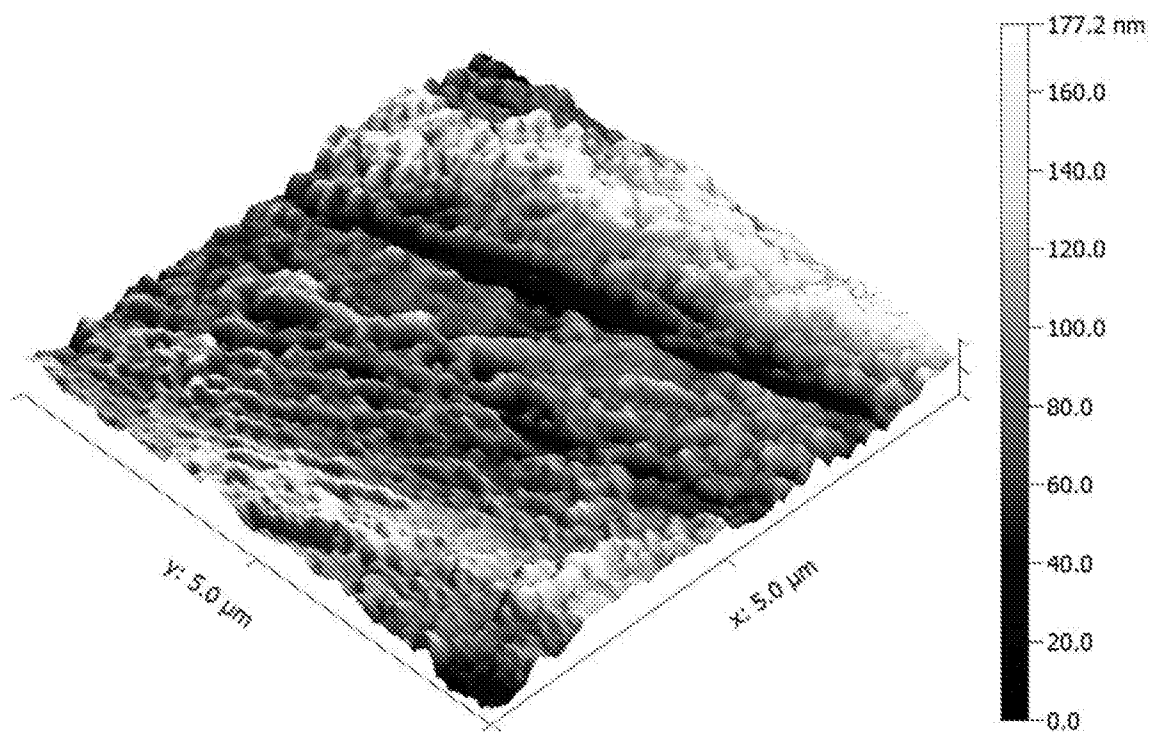


FIG. 3(A)

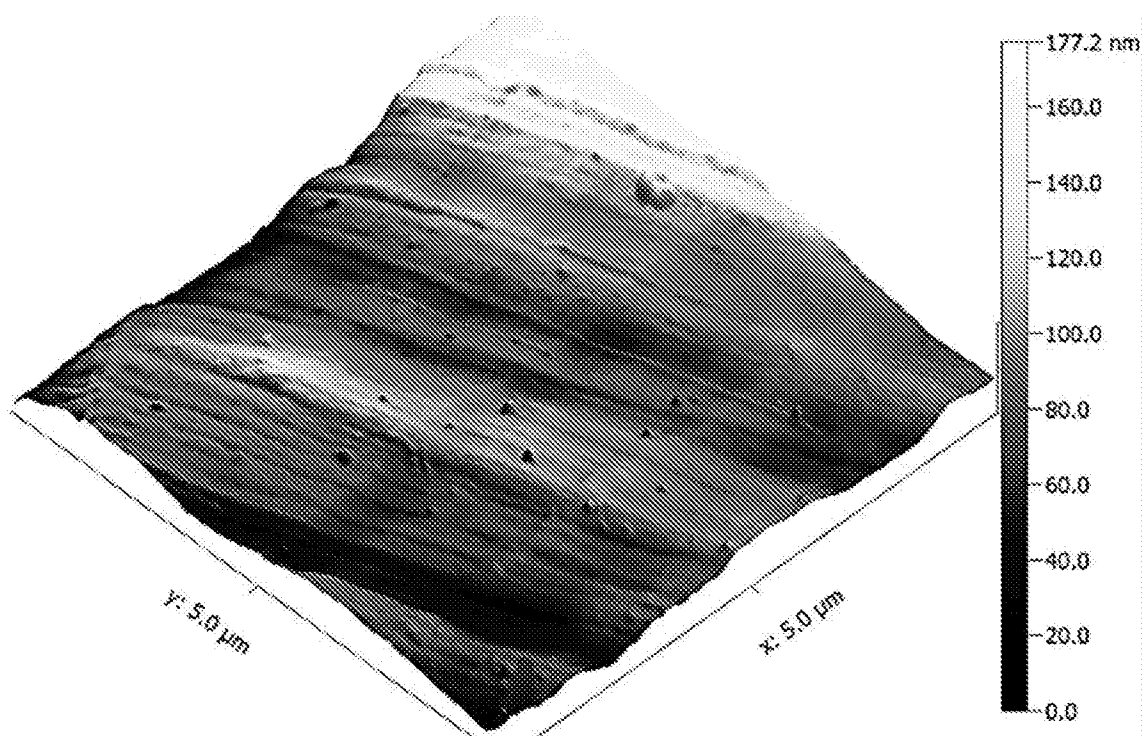


FIG. 3(B)

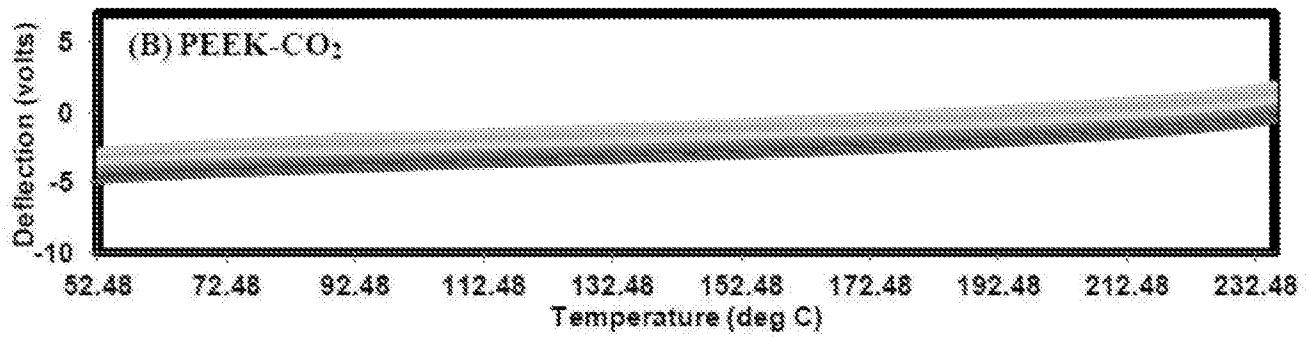


FIG. 4(A)

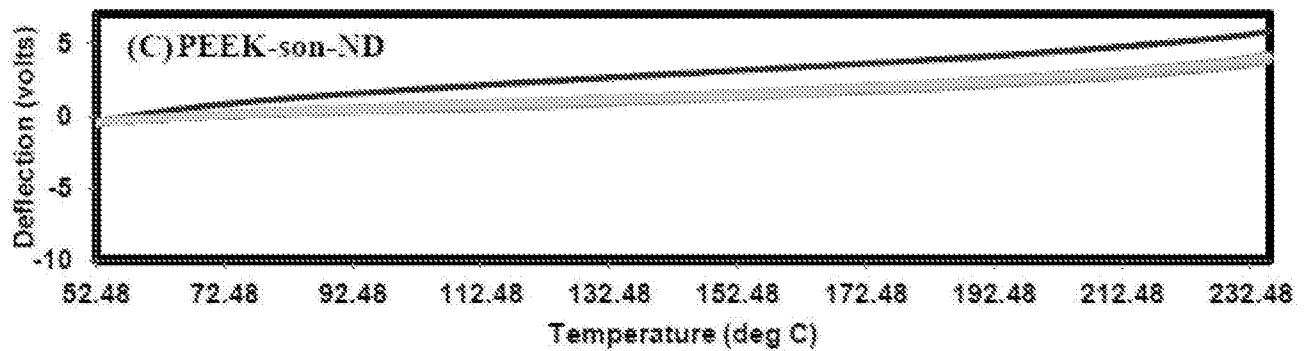


FIG. 4(B)

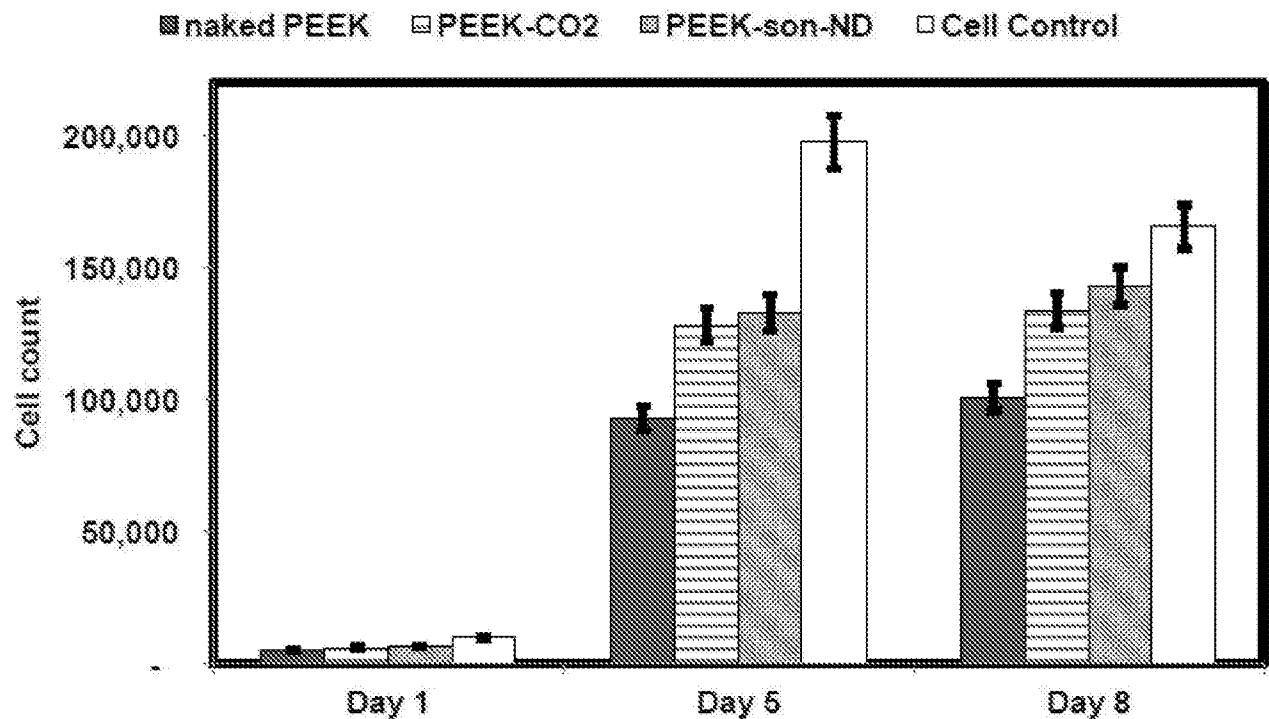


FIG. 5

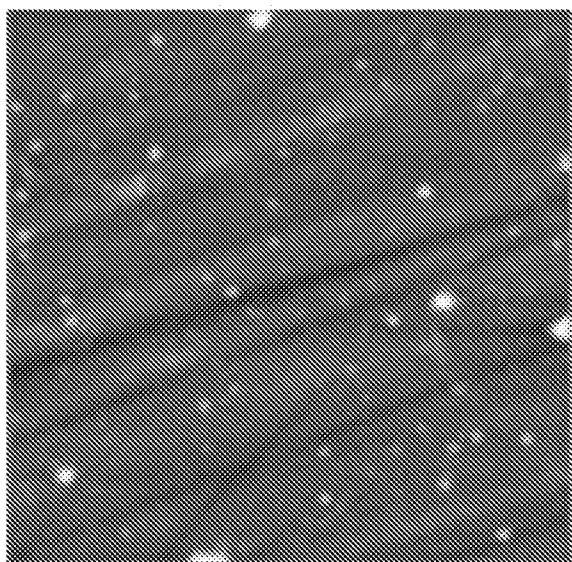


FIG. 6(A)

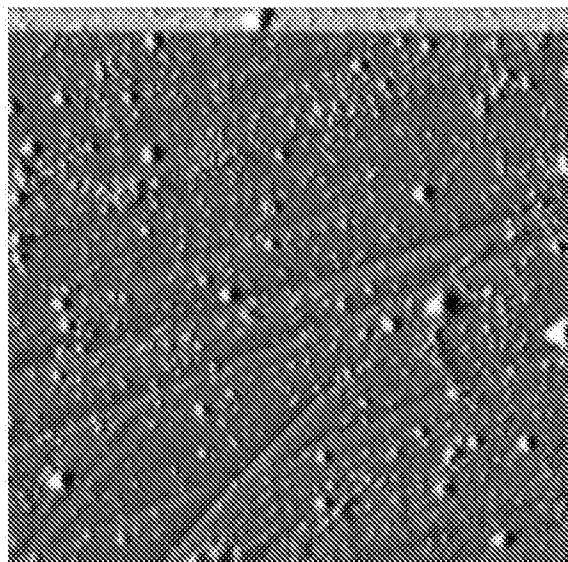


FIG. 6(B)

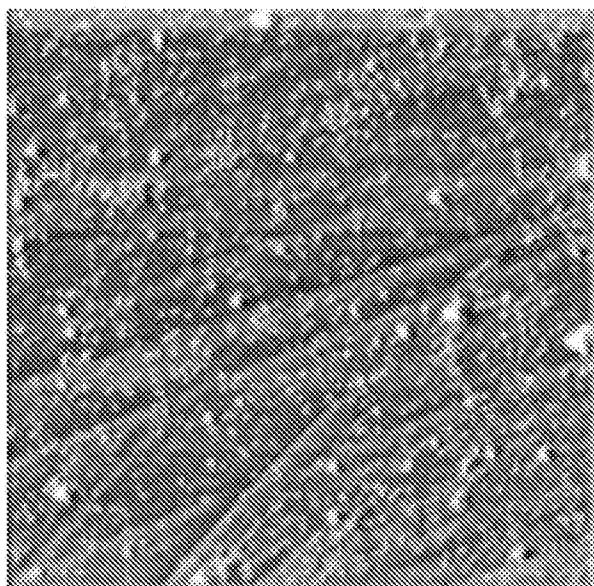


FIG. 6(C)

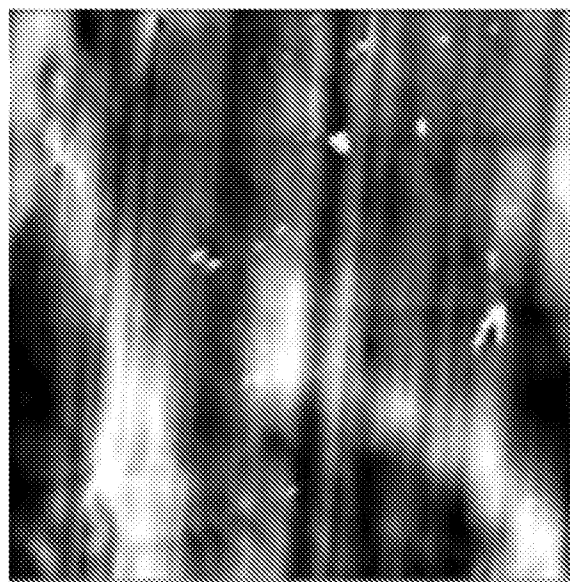


FIG. 7(A)

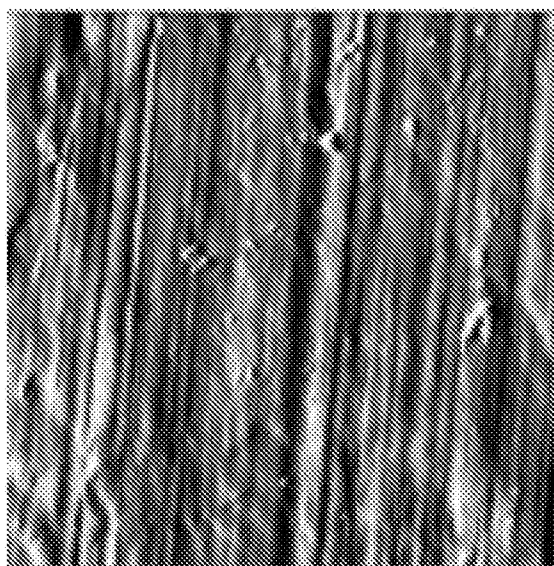


FIG. 7(B)

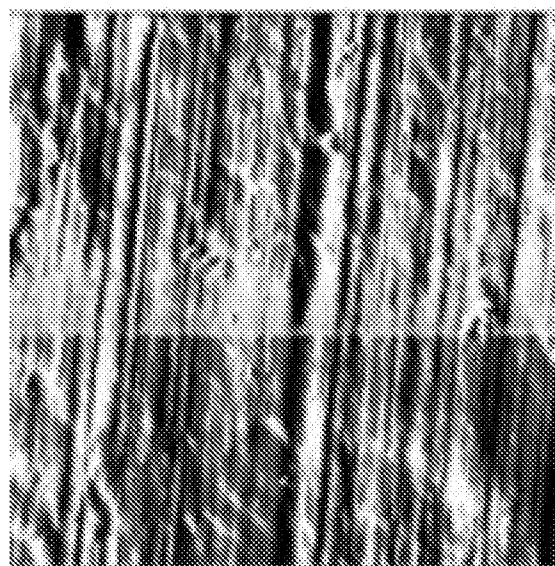


FIG. 7(C)

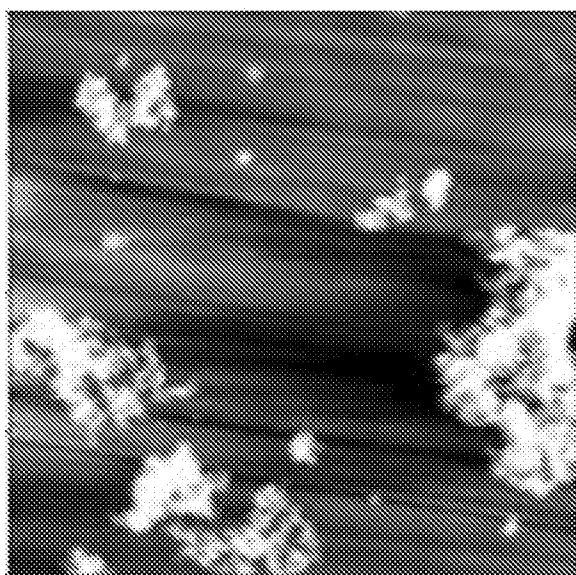


FIG. 8(A)

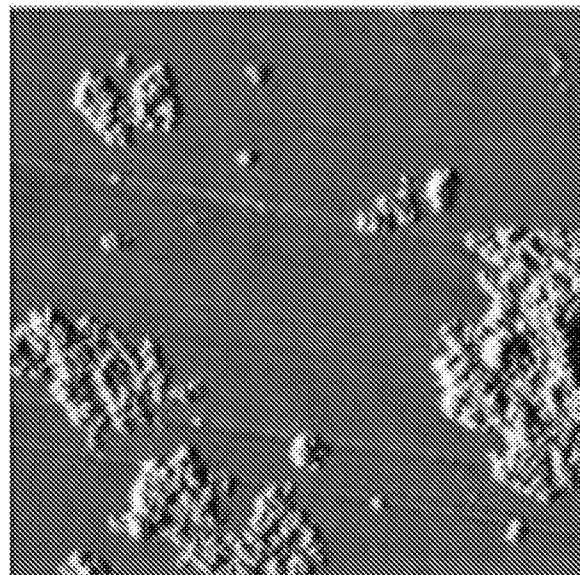


FIG. 8(B)

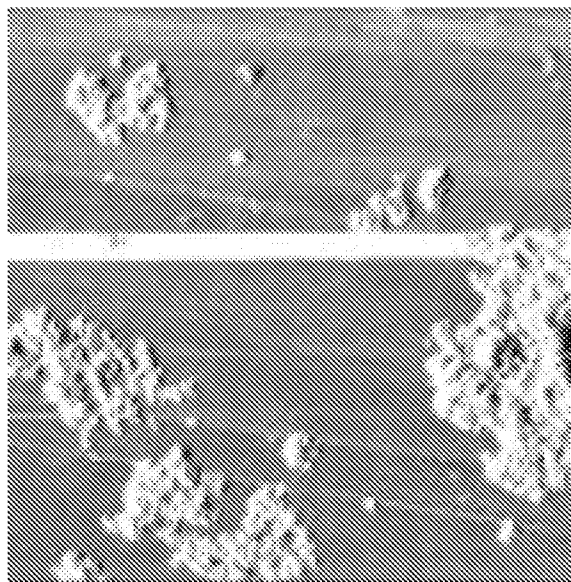


FIG. 8(C)

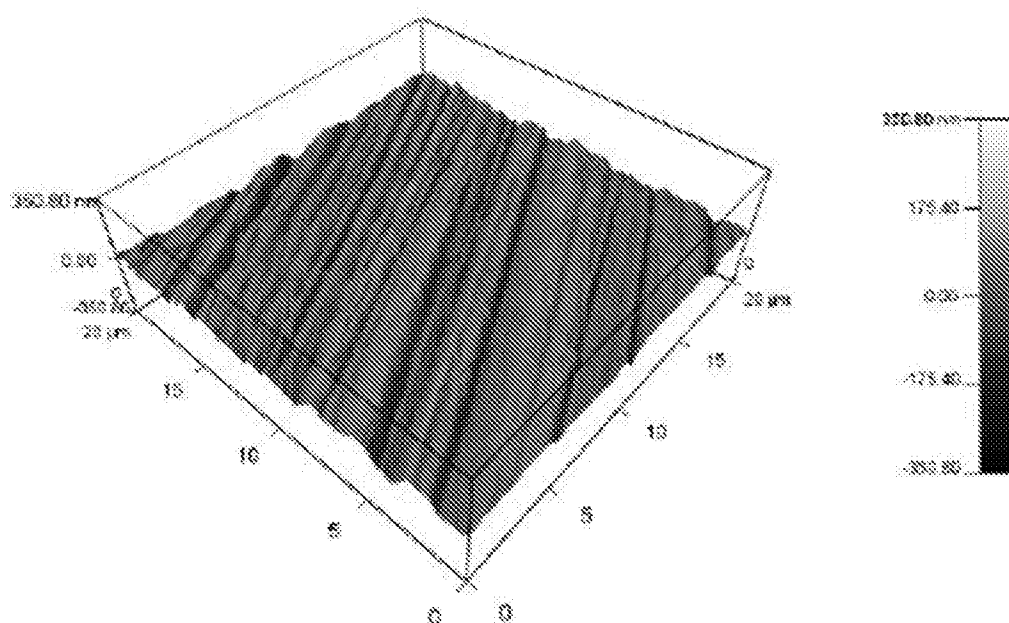


FIG. 9(A)

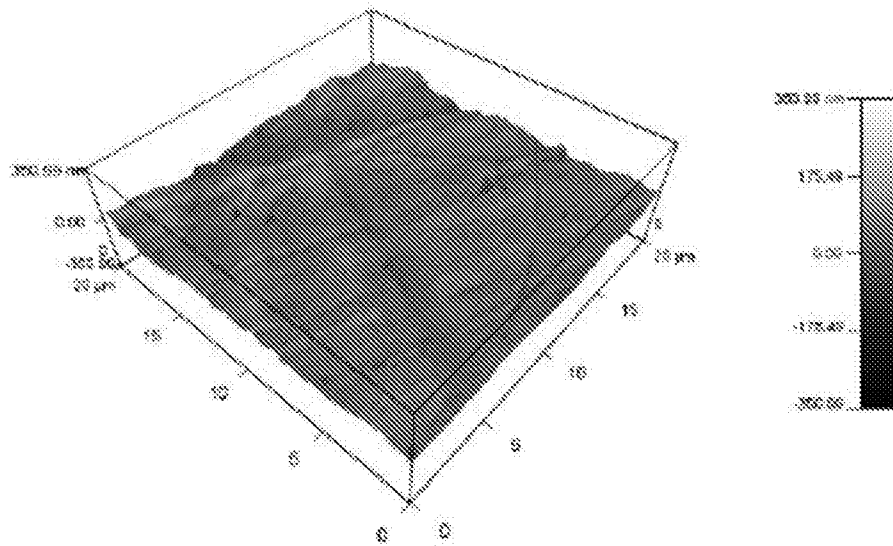


FIG. 9(B)

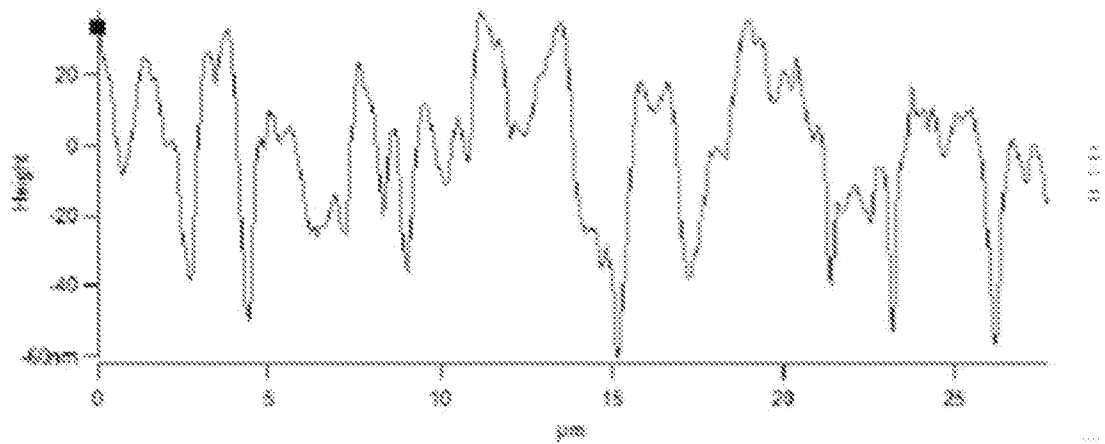


FIG. 10(A)

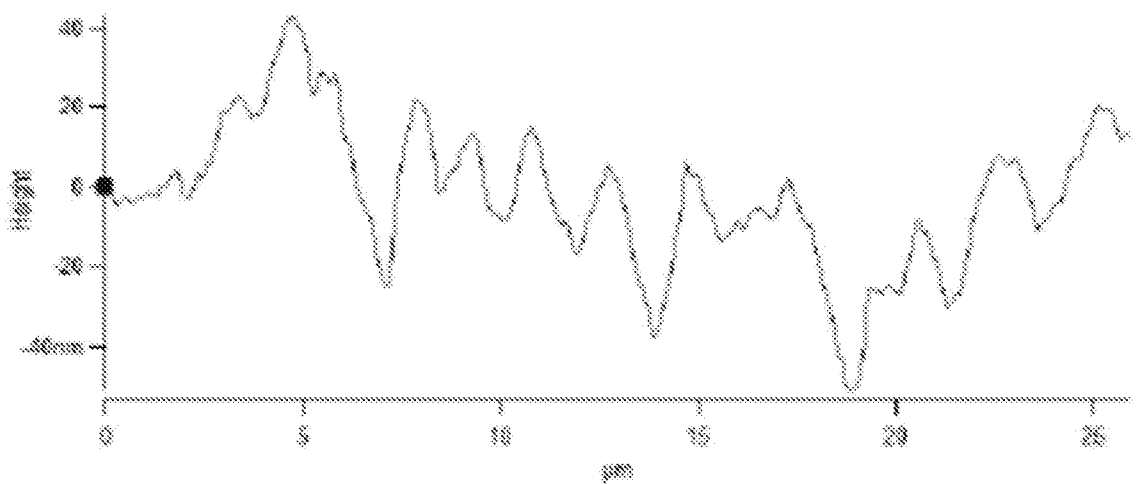


FIG. 10(B)

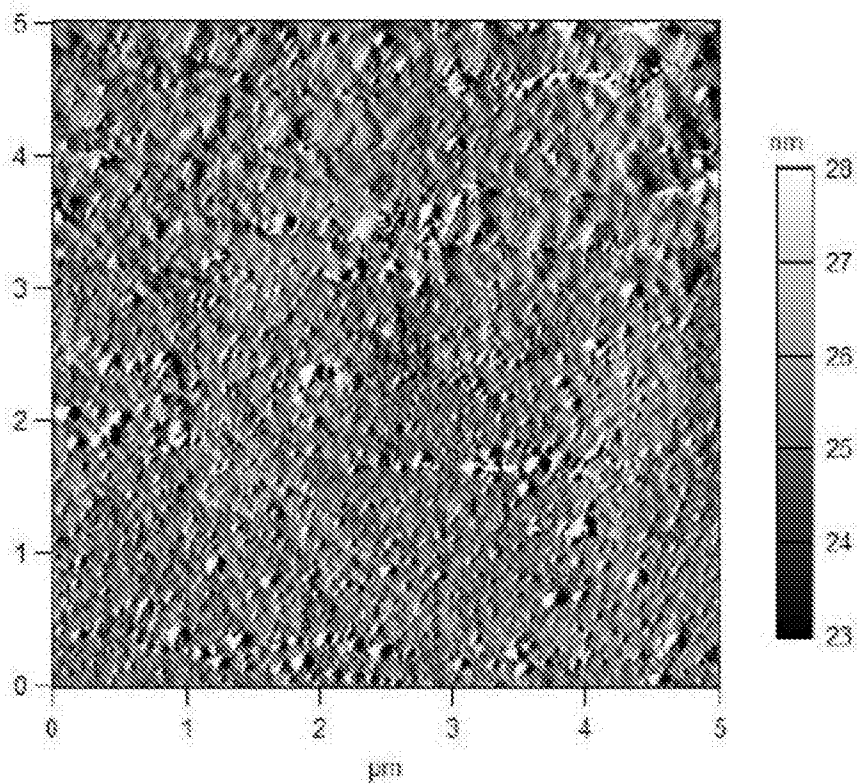


FIG. 11

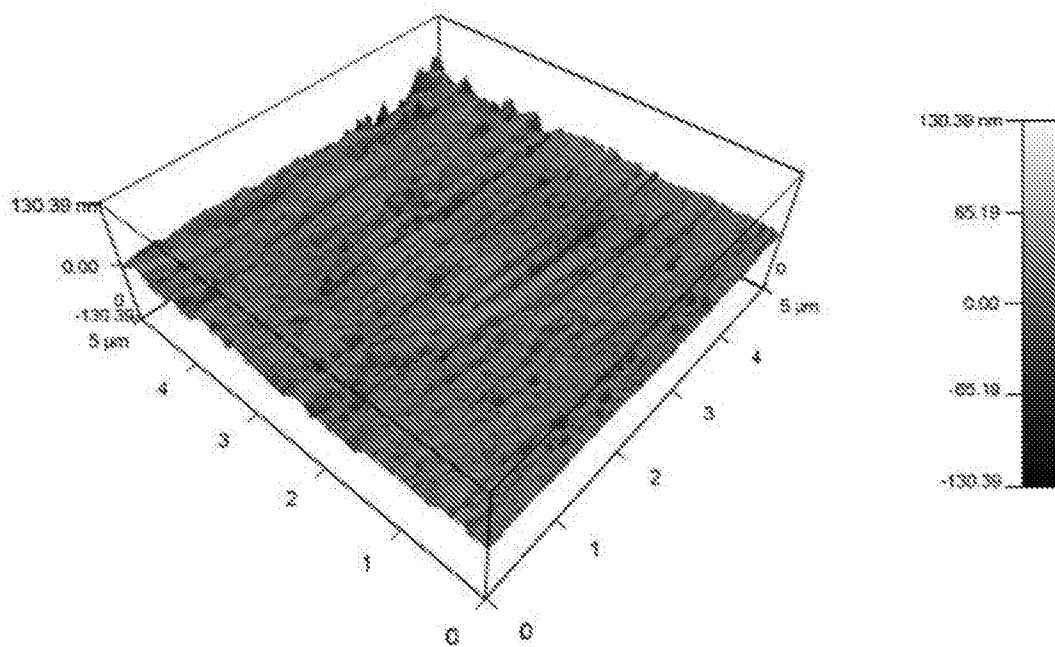


FIG. 12

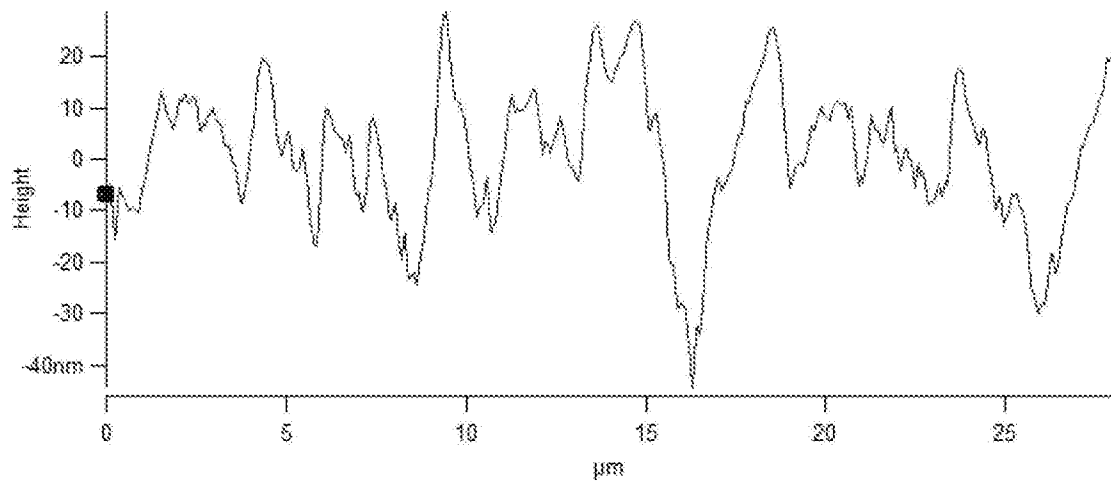


FIG. 13

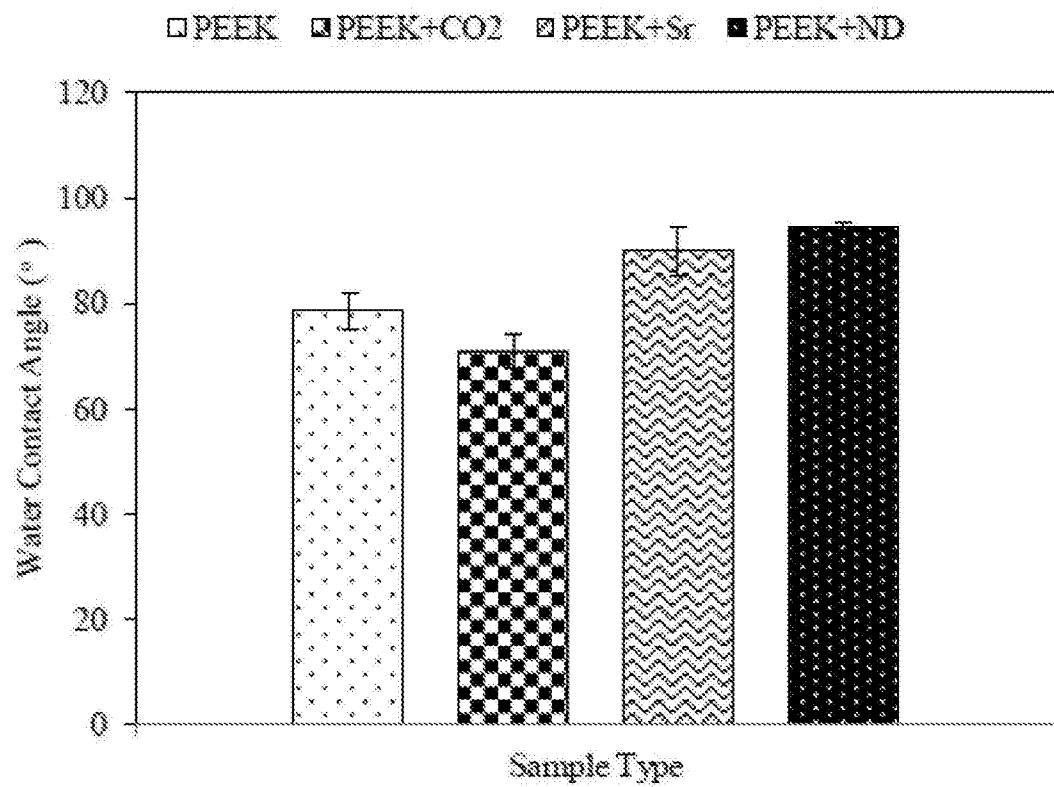


FIG. 14

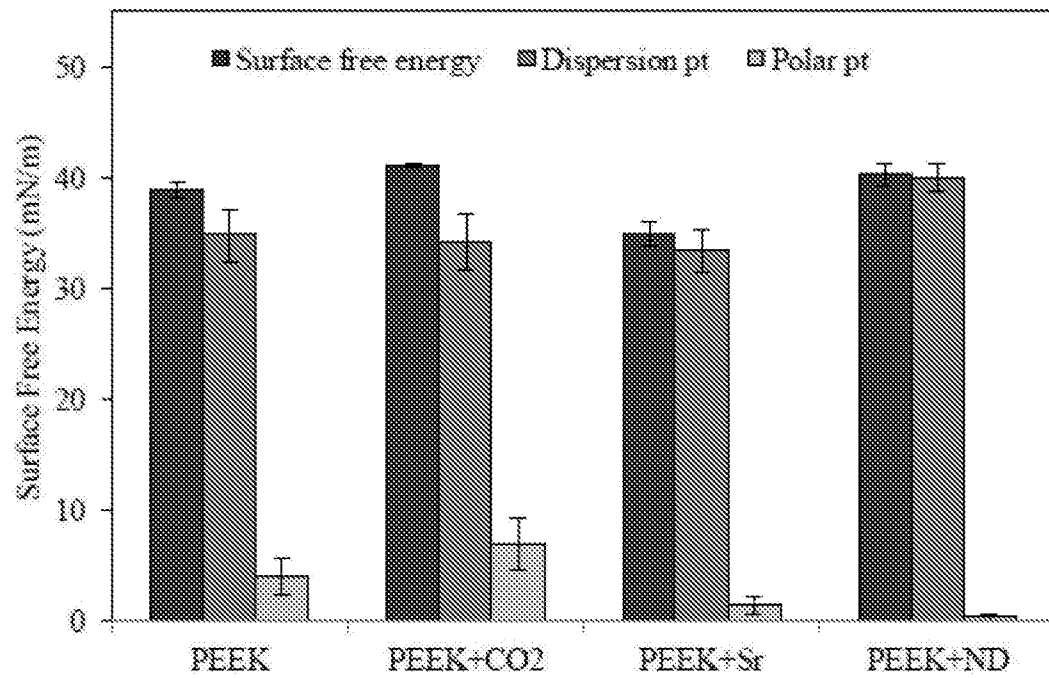


FIG. 15

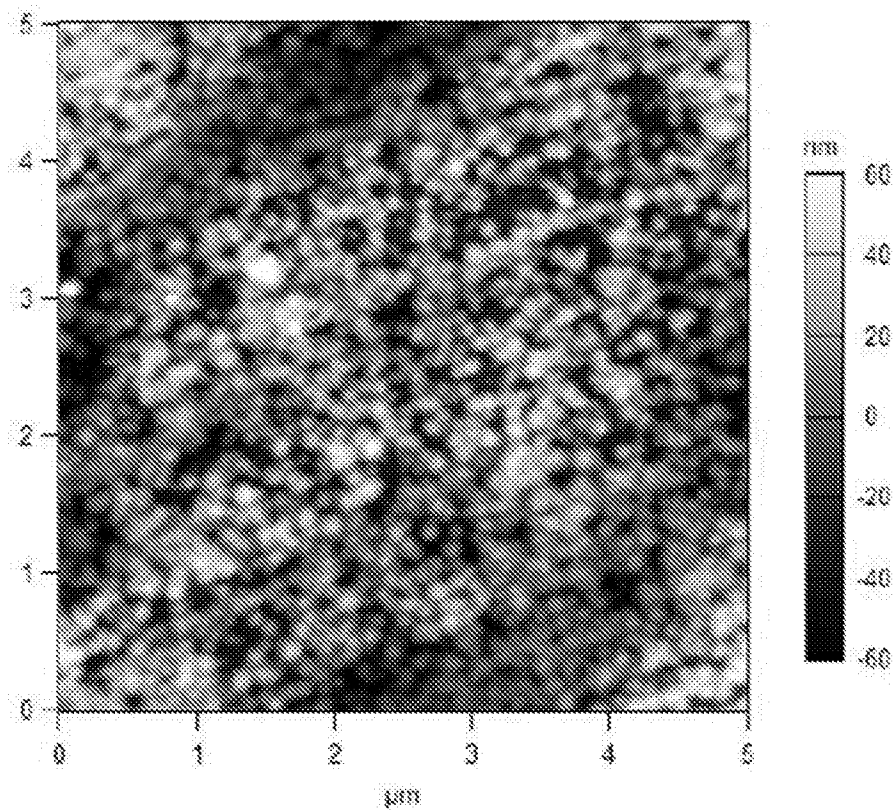


FIG. 16(A)

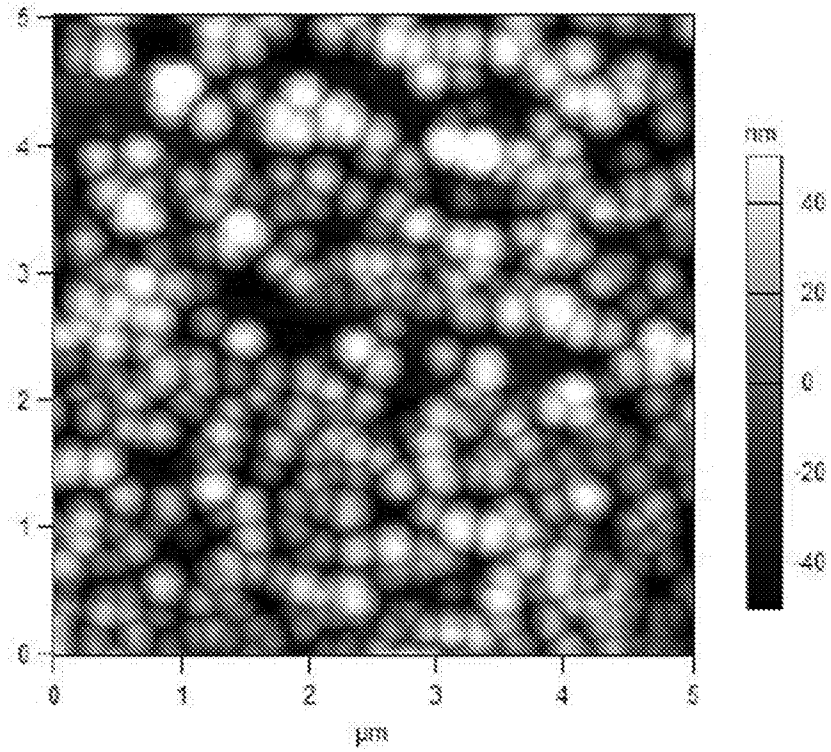


FIG. 16(B)

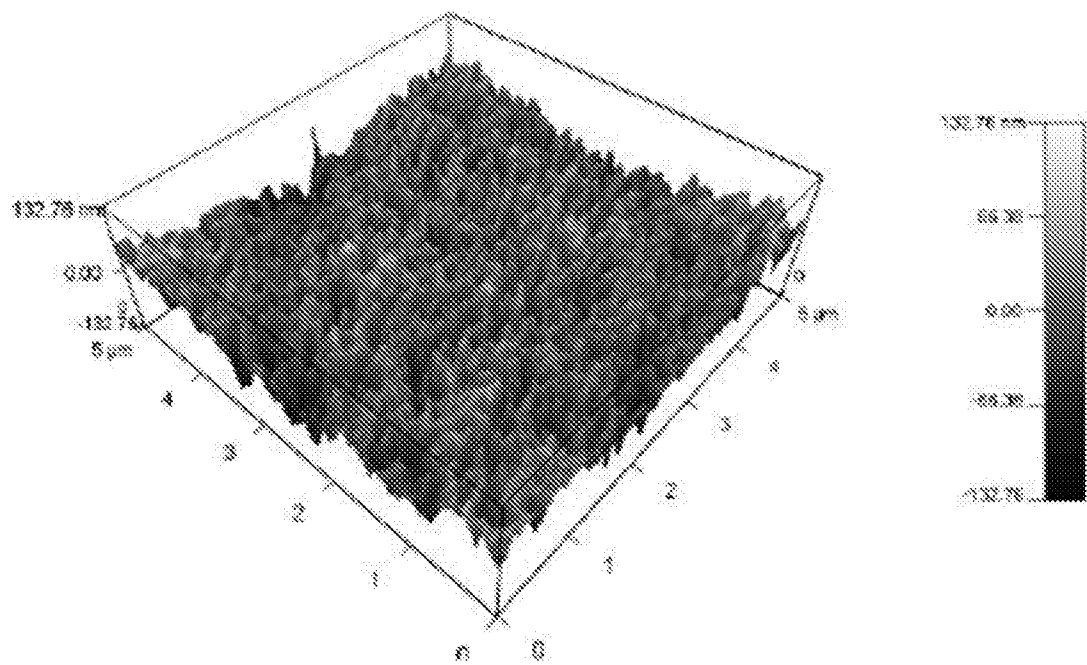


FIG. 17(A)

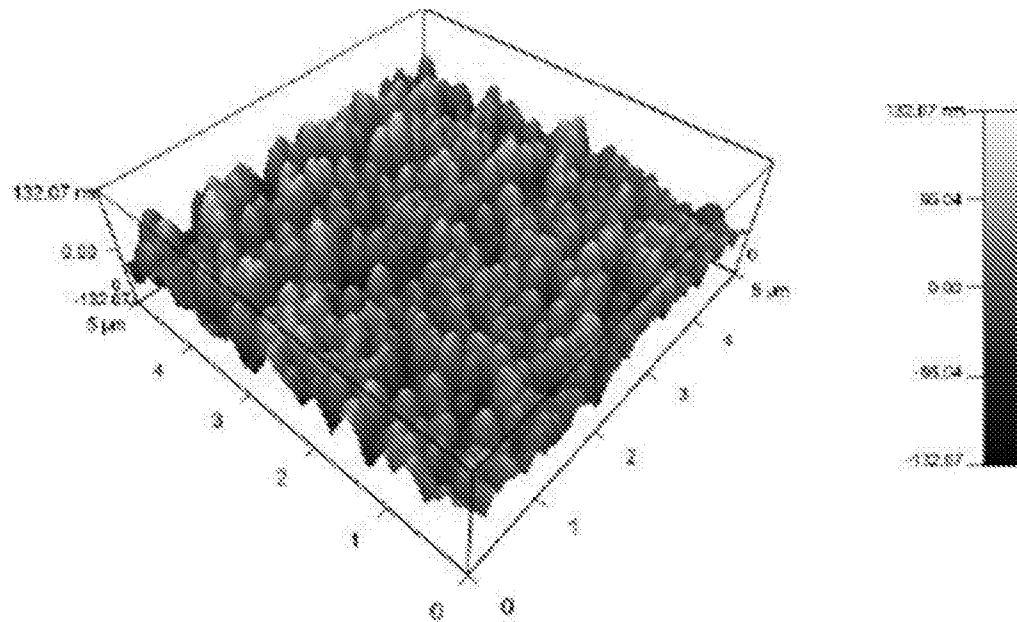


FIG. 17(B)

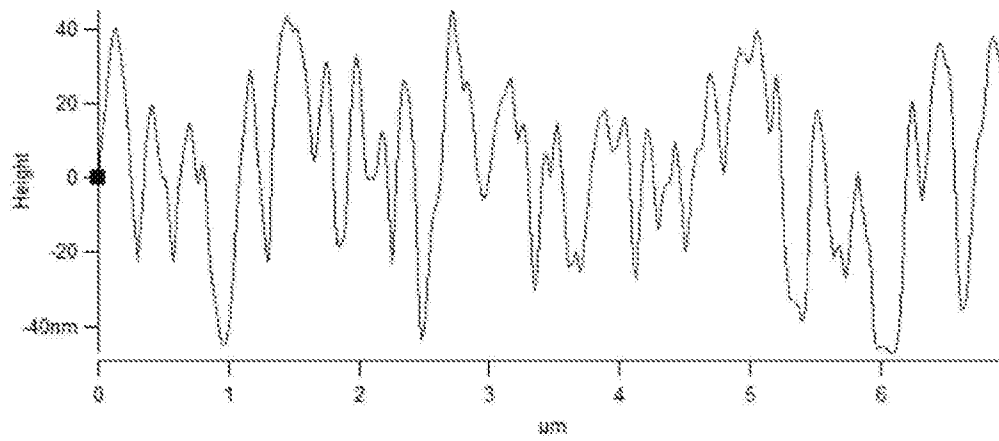


FIG. 18(A)

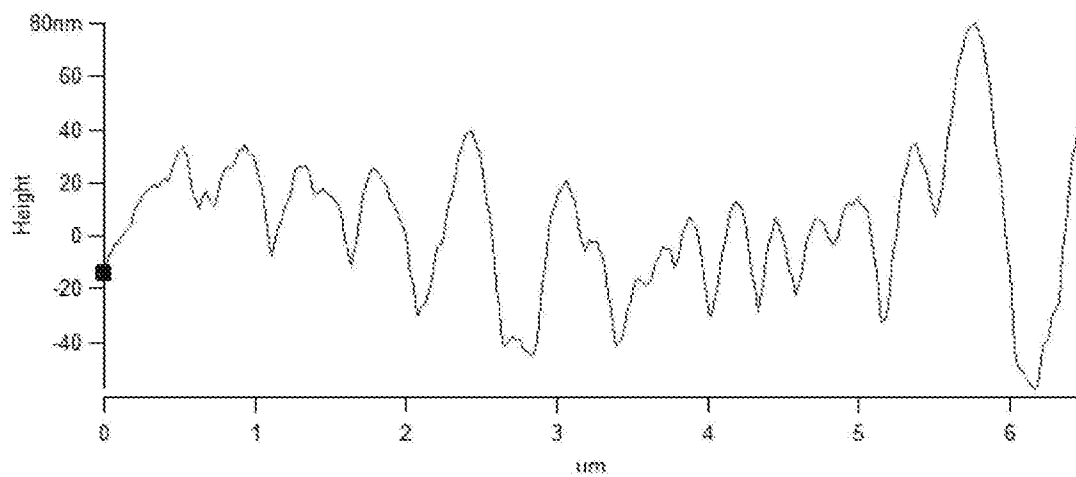


FIG. 18(B)

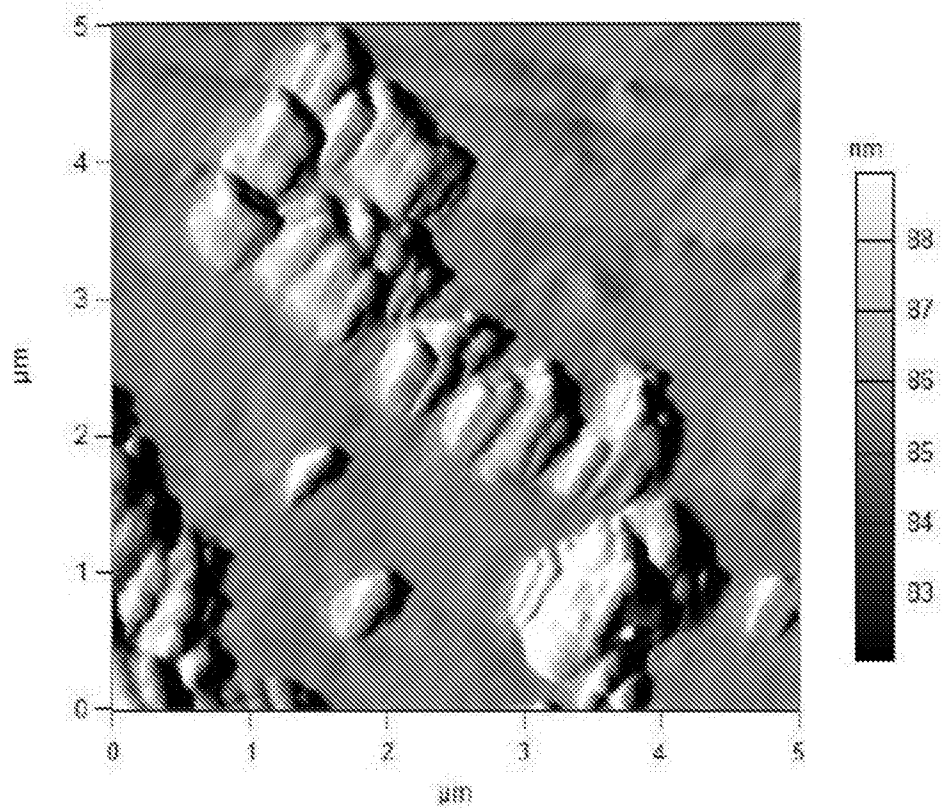


FIG. 19(A)

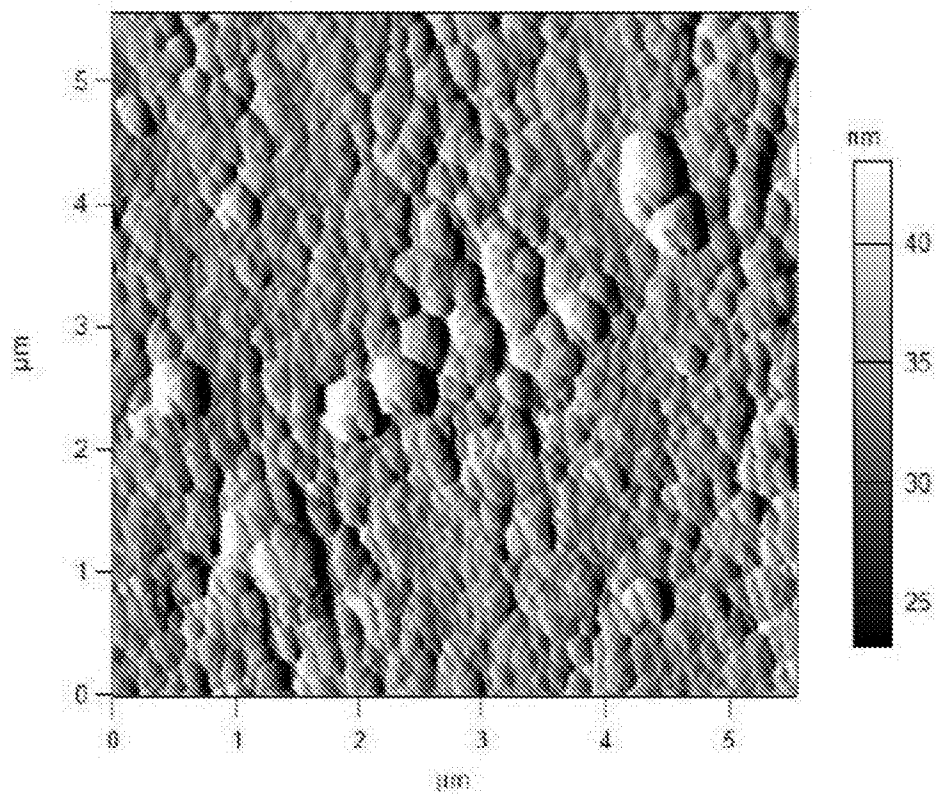


FIG. 19(B)

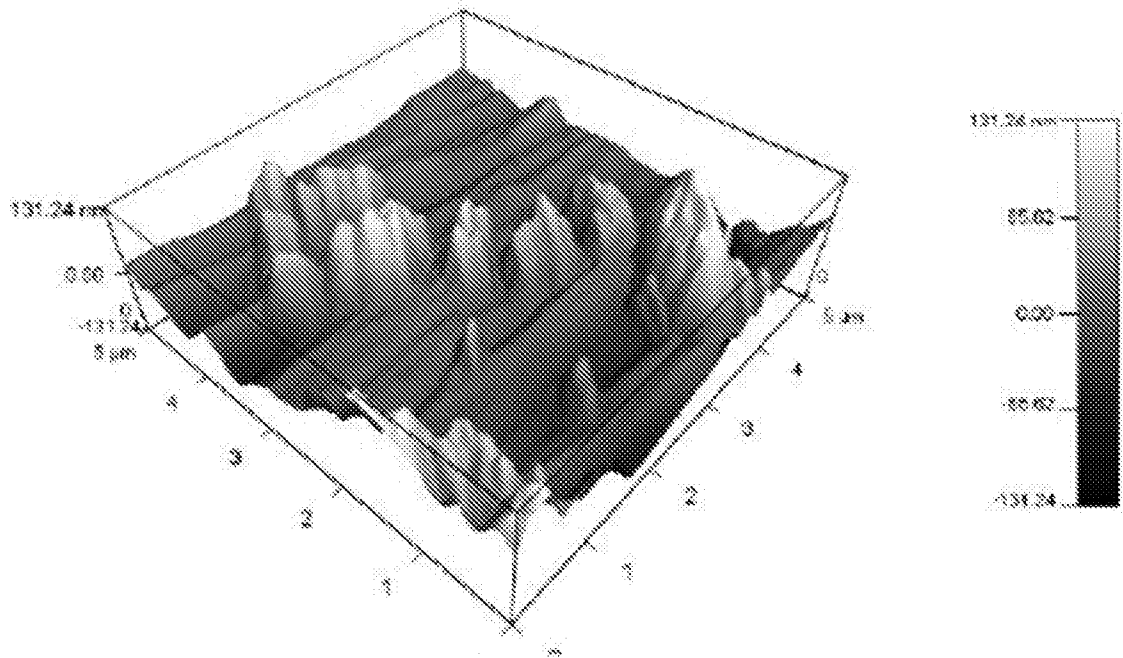


FIG. 20(A)

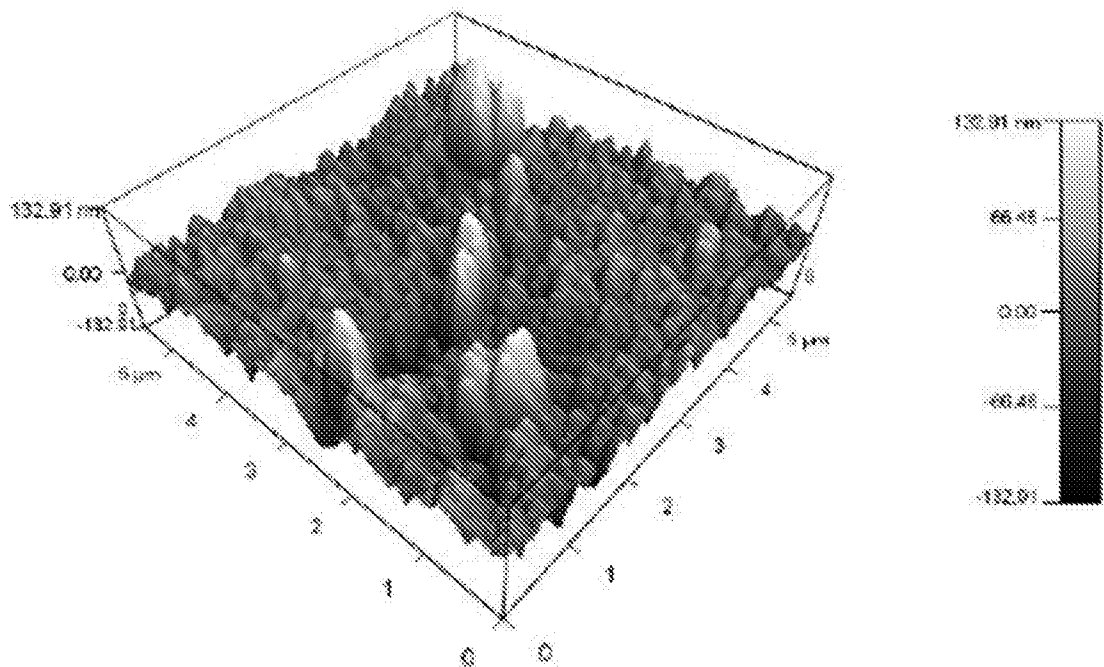


FIG. 20(B)

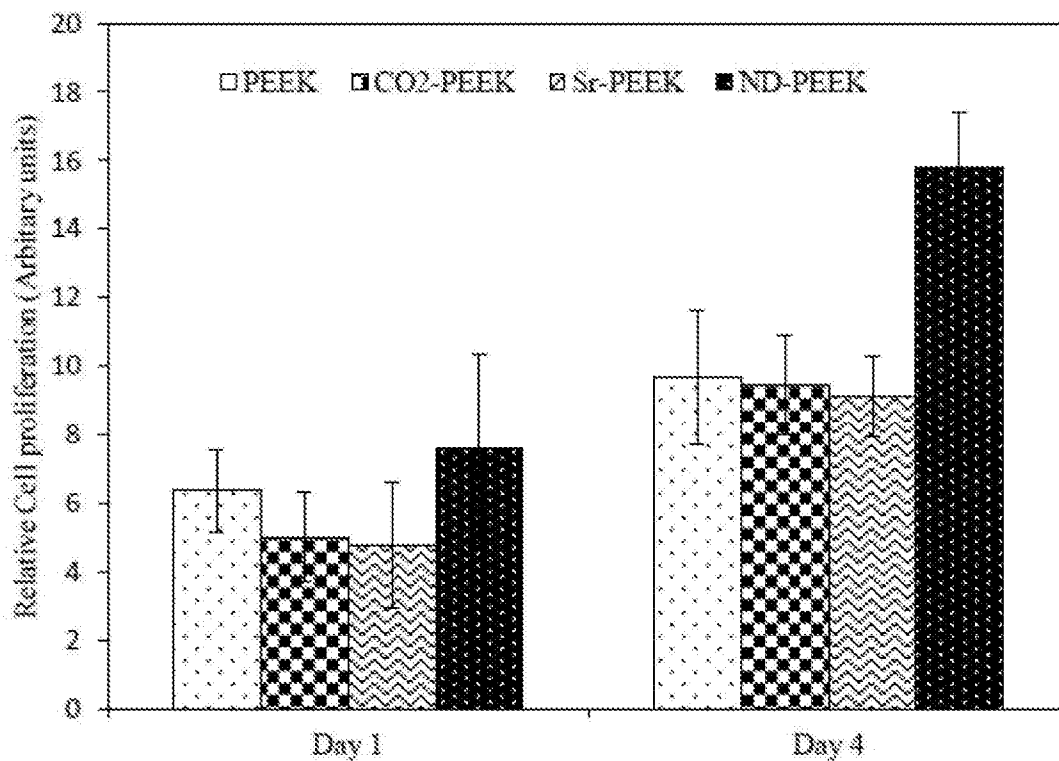


FIG. 21

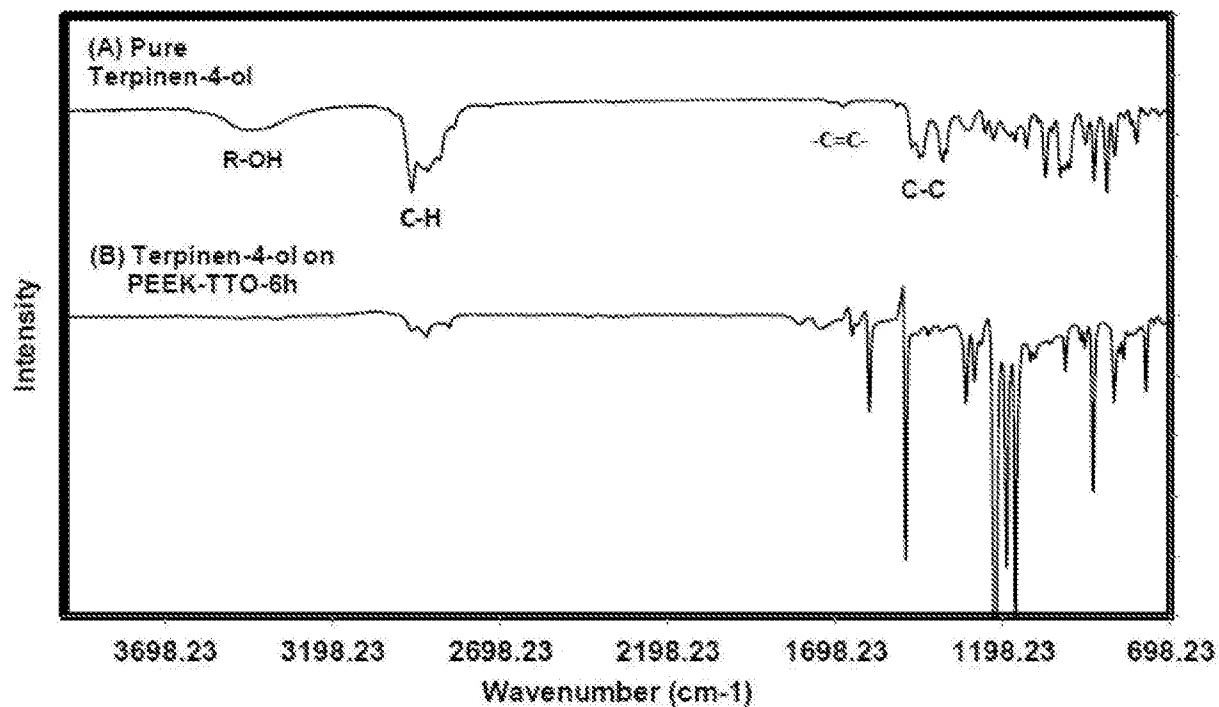


FIG. 22

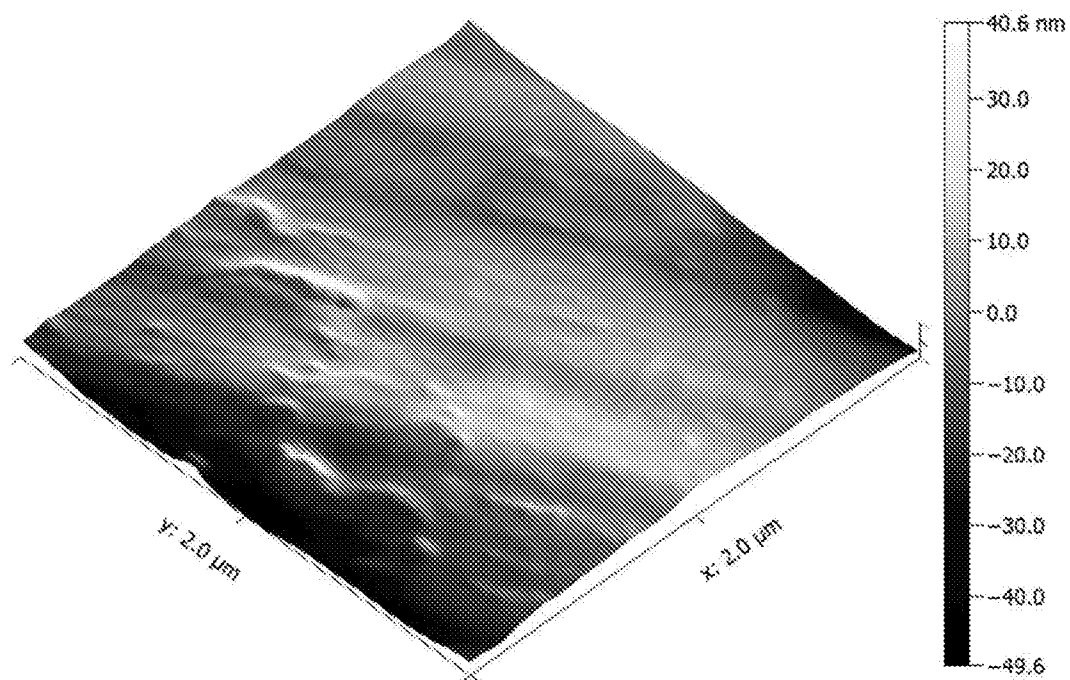


FIG. 23

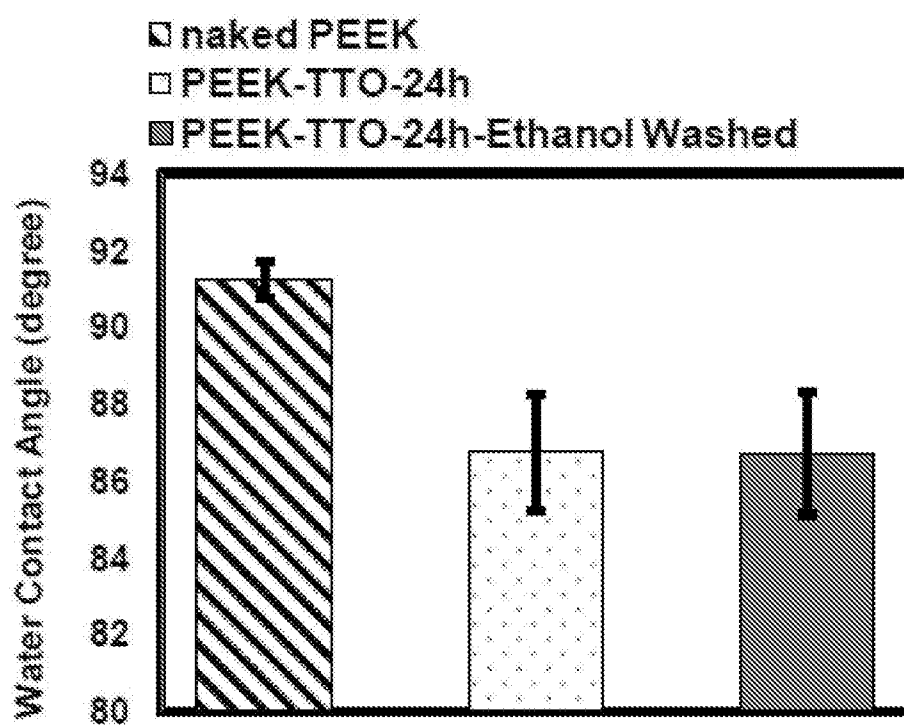


FIG. 24

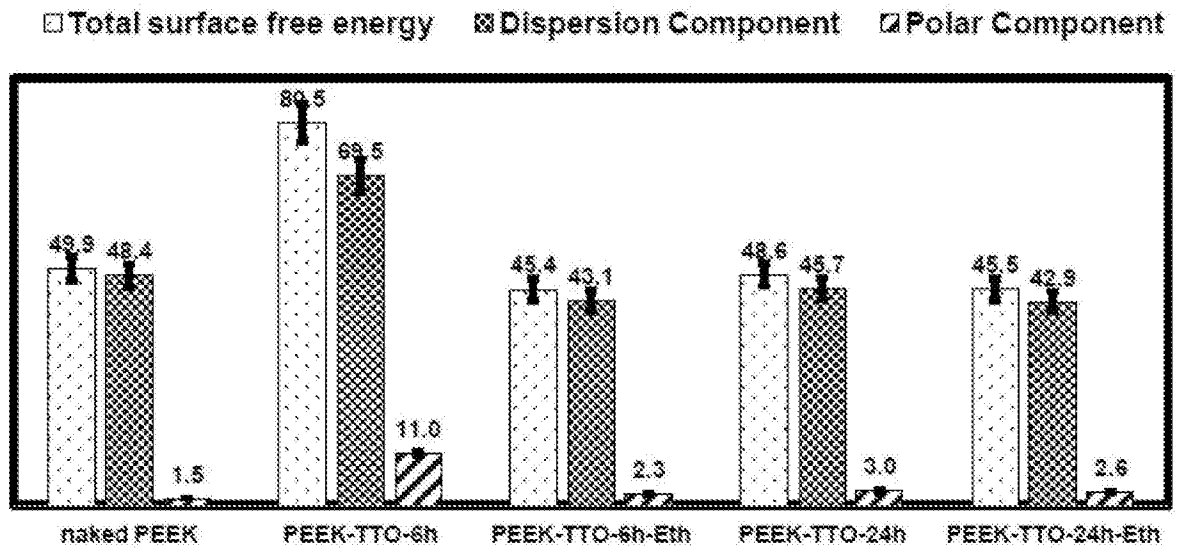


FIG. 25

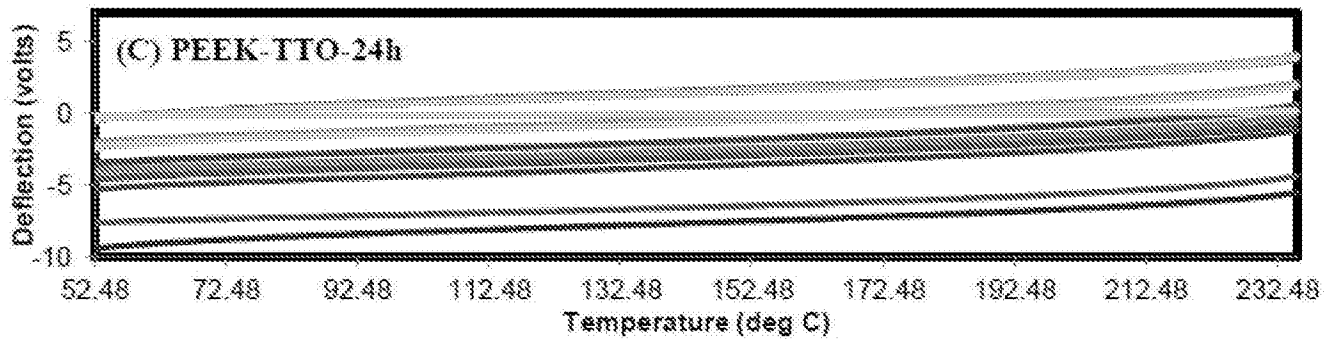


FIG. 26

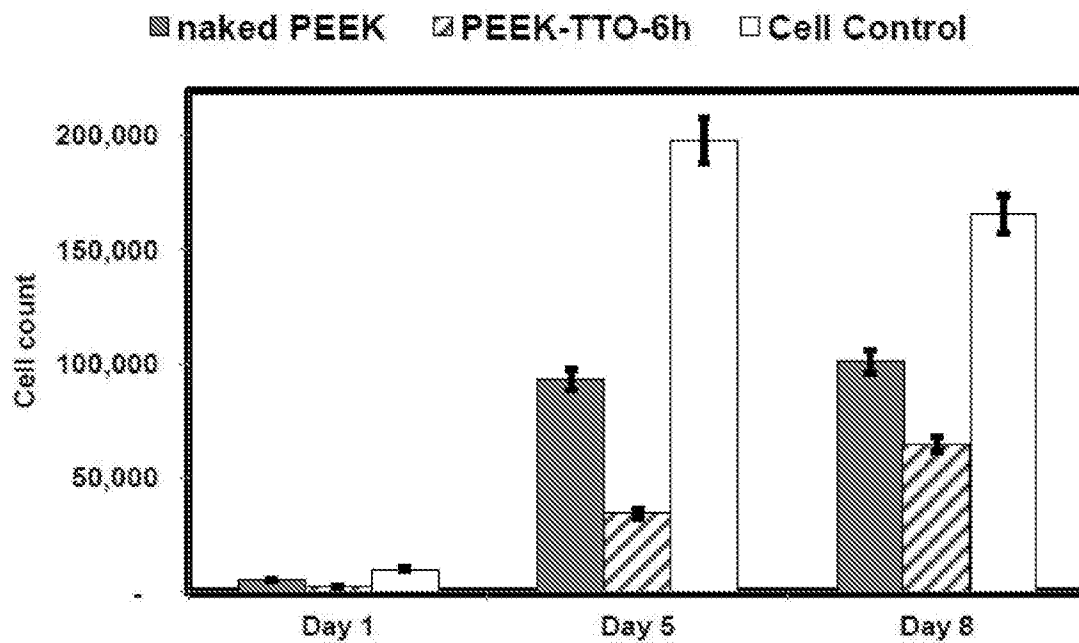


FIG. 27

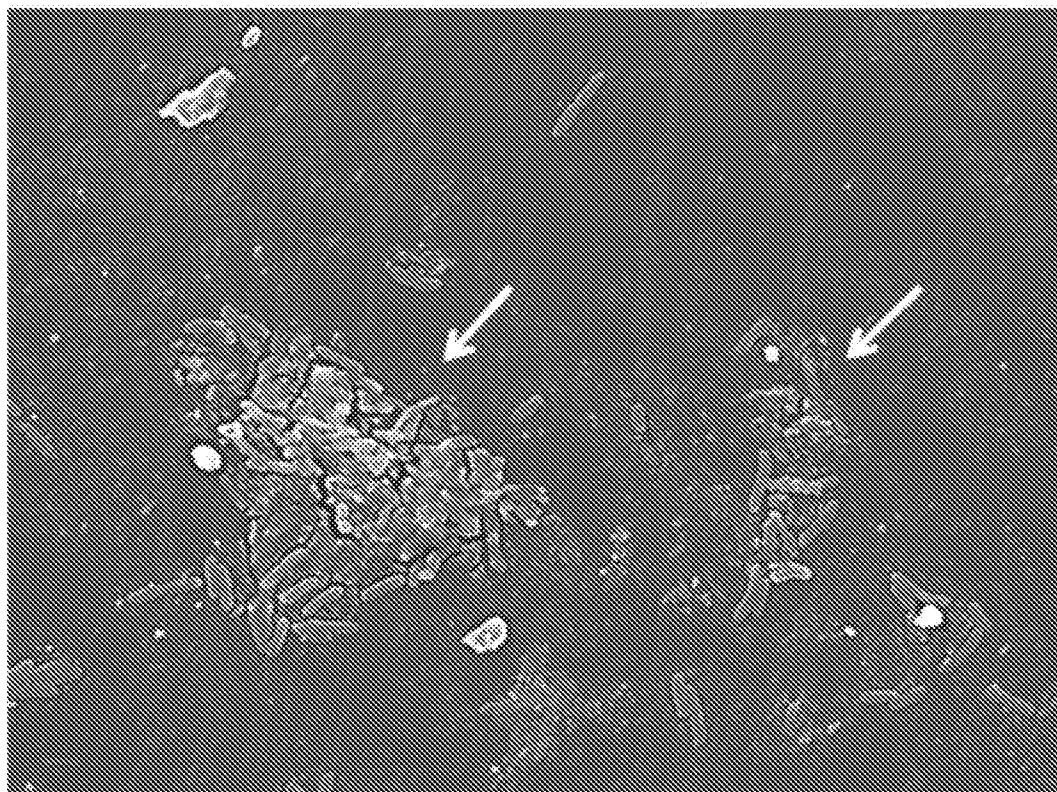


FIG. 28(A)

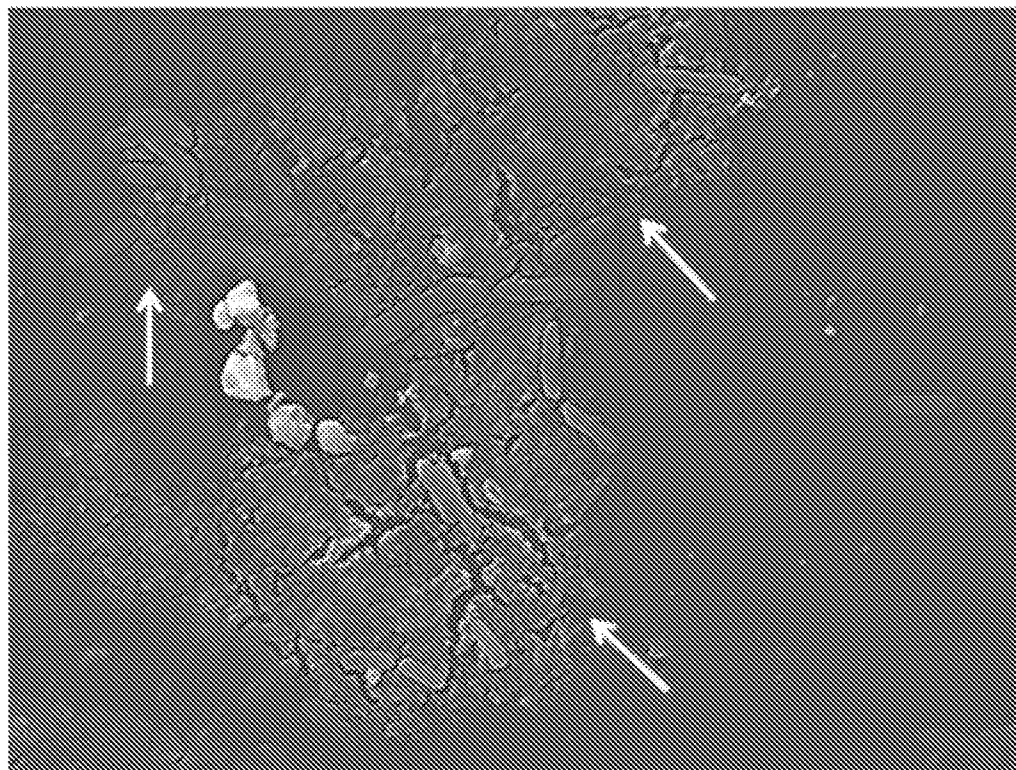


FIG. 28(B)

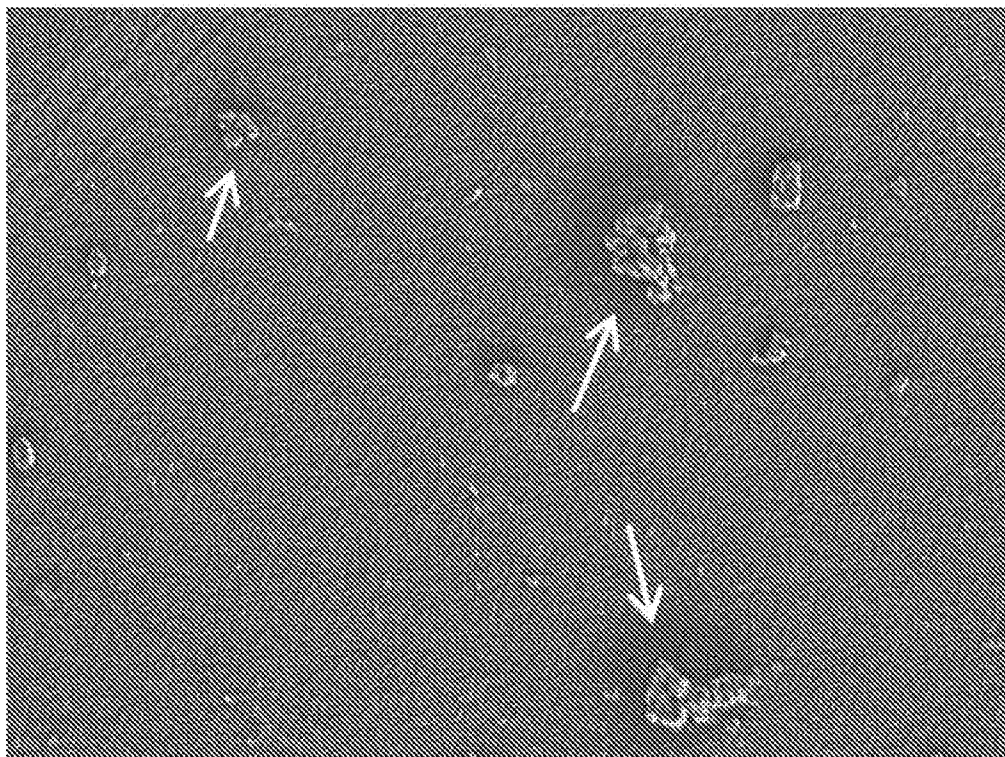


FIG. 29(A)

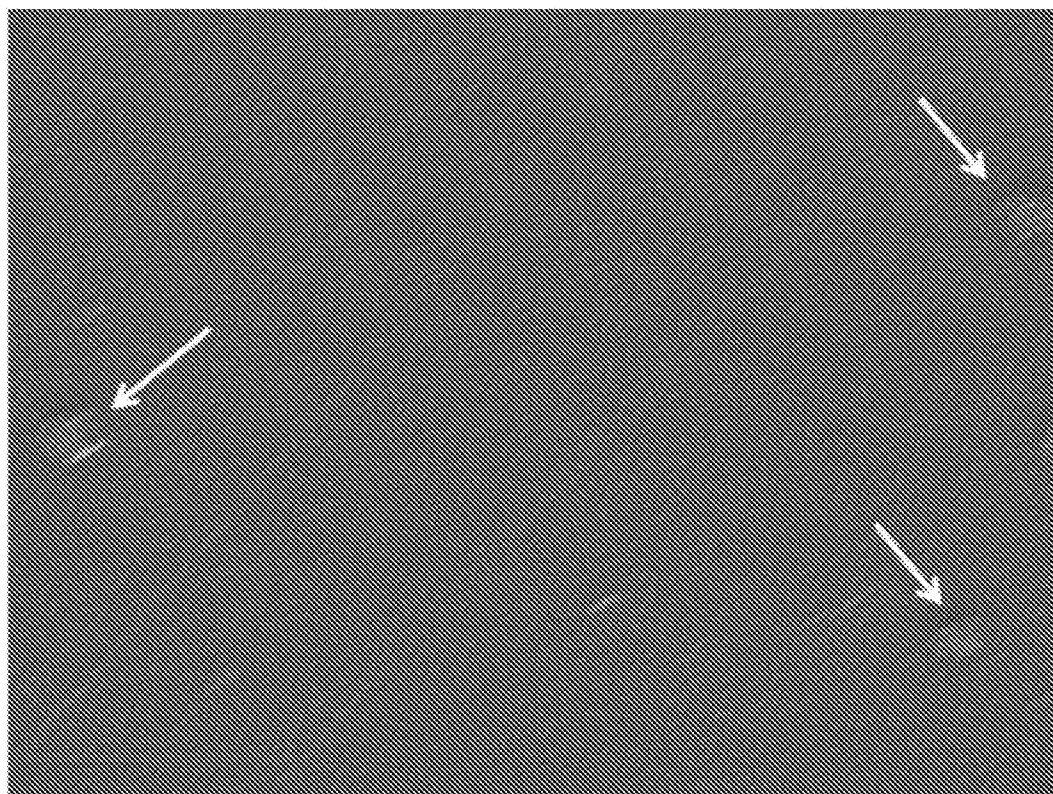


FIG. 29(B)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/000061

A. CLASSIFICATION OF SUBJECT MATTER

A61L 31/12 (2006.01) A61L 31/14 (2006.01) A61L 27/44 (2006.01) A61L 27/30 (2006.01)

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)

IPC: A61L 31/12, 31/14

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)

Chemical Abstracts (CAPLUS) and MEDLINE, keywords for concepts (1) supercritical carbon dioxide (2) polymers especially polyetheretherketone/PEEK (3) specific additives including nanodiamonds and terpinen-4-ol and (4) prostheses/implants; Espacenet, English Language Full text and Derwent Online Abstracts (WPIAP), concepts as above especially within IPC A61L/-; Inventor name searches in Espacenet, Google and Australian Internal Databases.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "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 "&" document member of the same patent family
Date of the actual completion of the international search 2 May 2016		Date of mailing of the international search report 02 May 2016
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Graeme Broxam AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832435

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2016/000061
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2001/0003007 A1 (CHINN et. al.) 07 June 2001 Abstract, paragraphs [0010], [0025], [0033]-[0039] Abstract, paragraphs [0010], [0025], [0033]-[0039]	1, 4-14, 33, 36, 45, 50-53 2-3
X Y	US 2003/0044514 A1 (RICHARD) 06 March 2003 Abstract, paragraphs [0018], [0021], [0034] Abstract, paragraphs [0018], [0021]	1, 4-14, 33, 36, 45, 50-53 2-3
X Y	WO 2003/045448 A1 (THE UNIVERSITY OF NOTTINGHAM) 05 June 2003 Abstract, page 2 lines 5-18, page 3 lines 5-20, Example 1. Abstract, page 2 lines 5-18, page 3 lines 5-20, Example 1.	1, 4-14, 33, 36, 45, 50-53 2-3
X Y	BAKER, K. C. et. al., "Supercritical Carbon-Dioxide Processed Resorbable Polymer Nanocomposites as Bone Graft Substitutes", Poster No. 1213, 56th Annual Meeting of the Orthopaedic Research Society (2010), retrieved from the Internet 1 April 2016: <www.ors.org/Transactions/56/1213.pdf> Methods, Results. Methods, Results.	1, 4-14, 33, 36, 45, 50-53 2-3
X Y A	KAZARIAN, S. G., "Polymer Processing with Supercritical Fluids", <i>Polymer Science, Ser. C</i> , Vol. 42, No. 1 (2000), pages 78-101. Part III: Implications of CO ₂ -induced Plasticisation of Polymers (ii) Impregnation and Dyeing Part III: Implications of CO ₂ -induced Plasticisation of Polymers (ii) Impregnation and Dyeing Abstract	1 2-3 1-53
X	GRAUSOVA, L. et. al., "Nanodiamond as Promising Material for Bone Tissue Engineering", <i>Journal of Nanoscience and Nanotechnology</i> , Vol. 9 (2009), pages 3524–3534. Abstract	48, 50, 52
X	WEHLING, J. et. al., "Bactericidal Activity of Partially Oxidized Nanodiamonds", <i>ACS Nano</i> , Vol. 8, No. 6 (2014), pages 6475-6483. Abstract	48, 50, 52
Y A	WO 2002/000275 A1 (VICTREX MANUFACTURING LIMITED) 03 January 2002 Abstract, examples Abstract, examples	2-3 15-32, 34-35, 37-44, 46-47
Y A	US 2008/0069852 A1 (SHIMP et. al.) 20 March 2008 Abstract, examples Abstract, examples	2-3 15-32, 34-35, 37-44, 46-47
X	ZHANG, Q. et. al., "Fluorescent PLLA-nanodiamond composites for bone tissue engineering", <i>Biomaterials</i> , Vol. 32 (2011), pages 87-94. Abstract	48, 50, 52
X	BAZAKA, K. et. al., "Plasma-assisted surface modification of organic biopolymers to prevent bacterial attachment", <i>Acta Biomaterialia</i> , Vol. 7 (2011), pages 2015–2028. 2. Plasma Modification; 5. Effect of material properties of the substrate: second column of page 2020.	49, 51, 53
Form PCT/ISA/210 (fifth sheet) (July 2009)		

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Claims 1-49 and in part 50-53 are directed to biocompatible materials, and methods of making and using them.. The feature of plasticising and impregnating the materials using supercritical carbon dioxide is specific to this group of claims.
- Claims 48 and in part 50 and 52 are directed to biocompatible materials. The feature of impregnating at least the surface of the biocompatible materials with nanodiamonds is specific to this group of claims.
- Claims 49 and in part 51 and 53 are directed to biocompatible materials. The feature of impregnating at least the surface of the biocompatible materials with terpinene-4-ol is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is the provision of biocompatible materials with a material impregnated onto at least its surface. However this feature does not make a contribution over the prior art because it is disclosed in, for example, D1 cited in this report.

Therefore in the light of this document this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2016/000061	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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		CA 2412492 A1	03 Jan 2002
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			
Form PCT/ISA/210 (Family Annex)(July 2009)			

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2016/000061	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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		WO 2007084725 A2	26 Jul 2007
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			