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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 11-037516-00011-0000

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Tra. 2 PATENTES Eve: 1 REGDEPOSITO
Act. 422 APELARECUR Folios: 71

Clarke

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **11-037516**
Solicitud de patente a nombre de **UNIVERSIDAD
AUTÓNOMA DE OCCIDENTE**

Yo, SILVANA MARIA LOPEZ DIAZ, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, de la sociedad **UNIVERSIDAD AUTÓNOMA DE OCCIDENTE**, domiciliada en Cali Colombia, solicitante de la patente de la referencia, interpongo recurso de reposición contra la resolución **No. 86133** emitida el **26 de diciembre de 2013** por el Superintendente de Industria y Comercio, por medio de la cual se niega el privilegio de patente para la solicitud contenida en el expediente de la referencia.

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio el cual consta de tres (3) reivindicaciones dirigidas al biocompuesto cerámico para regeneración ósea definido por una fase sólida con intervalos específicos de tres componentes, una fase líquida de quitosano y caracterizado además porque la relación de fase sólida a fase líquida es de 0.67. Sobre este particular me permito decir que se presenta tasa por modificación de la solicitud pero no por reivindicaciones adicionales, toda vez que el nuevo pliego reivindicatorio contiene tan solo 3 cláusulas las cuales no amplían de ninguna manera la materia objeto de la presente invención y se basan en las antiguas reivindicaciones 1 a 4 presentadas con la anterior respuesta.

2. FUNDAMENTOS DEL RECURSO

En relación con los argumentos presentados por ese despacho en la resolución impugnada **No. 86133** emitida el **26 de diciembre de 2013**, es necesario señalar que carecen de un enfoque adecuado de conformidad con las normas,

1/71

jurisprudencia y reglas técnicas que existen para evaluar la altura inventiva de una invención.

1.1 Violación de los artículos 16 y 18 de la Decisión 486 por una indebida aplicación de la norma basada en un error de interpretación de la misma.

En la resolución impugnada se pone de manifiesto que la diferencia entre la presente invención y el estado de la técnica, específicamente el documento denominado D1 (EP 0329098) consiste en que dicha anterioridad no menciona el β -fosfato tricálcico y que la diferencia con D2 (Us2011/00004219) está en que dicho documento no menciona el óxido de zinc, óxido de calcio y una solución de quitosano.

Se dice además que el efecto técnico alcanzado con la inclusión del β -fosfato tricálcico permite la bioabsorción del implante y promueve el crecimiento del tejido óseo de forma más eficiente y se plantea el problema como la modificación de la composición divulgada en D1 para que se realicen procesos de bioabsorción del implante y recuperación del tejido óseo de manera eficiente.

Consecuentemente, considera el examinador que un experto en la materia *"...incluiría el β -fosfato tricálcico de D2 en la composición de la anterioridad D1, esperando con certeza lograr el producto, como la de la reivindicación 1 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura"*.

Adicionalmente, en la resolución impugnada se establece que *"Sin embargo, el empleo β -fosfato tricálcico como fuente de calcio y fosfato diferentes a las apatitas para recuperación del tejido óseo ya está divulgado en el documento D2 que se refiere a composiciones como soporte para tejido duro (Pag. 2, Párr. 15, Pag. 4, Párr. 43), siendo conocido para la persona versada en la materia que que [sic] el β -fosfato tricálcico es bioabsorbible y puede ser sustituto para una hidroxiapatita autóloga y/o sintética"*.

En consecuencia, una persona normalmente versada en la materia incluiría el β -fosfato tricálcico de D2 en la composición de la anterioridad D1, esperando con certeza lograr el producto, como la de la reivindicación 1 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

Puesto que las reivindicaciones dependientes 2 a 4 no mencionan características adicionales, se considera que tales reivindicaciones tampoco tienen nivel inventivo.

Es oportuno aclarar que, si bien el documento D1 no indica el empleo de β -fosfato tricálcico en conjunto con el óxido de zinc, óxido de calcio y la solución de quitosano, esta Oficina reitera que el documento D2 menciona el empleo β -fosfato tricálcico como fuente de calcio y fosfato diferente a las apatitas para recuperación del tejido óseo, en donde para la persona versada en la materia se tiene que el β -fosfato tricálcico es absorbible comparado con la hidroxiapatita, sea esta hidroxiapatita de origen biológico o sintética, y que el empleo de β -fosfato tricálcico promueve la formación de nuevo hueso no solo sobre los implantes sino también en los poros creados en poco tiempo sin la necesidad de tener tejido autólogo". Adicionalmente se tiene que D1 menciona un ejemplo dentro de los intervalos de concentración del óxido de zinc, óxido de calcio, solución de quitosano y porcentaje de la fuente de calcio y fosfato inorgánico sugeridos por la presente solicitud como composición para la regeneración ósea, sin empleo de óxido de magnesio (Pag. 3, Tabla. 1, ejemplo 3), por lo que es de esperarse para la persona versada en la materia que el cambio de hidroxiapatita por el β -fosfato tricálcico produzca el efecto esperado de bioabsorción."

90

En relación con esta argumentación consideramos que la resolución No. 86133 del 26 de diciembre de 2013 que se impugna se encuentra viciada de nulidad debido a que por un lado, no se ha fundamentado debidamente la falta de nivel inventivo con base al documento citado D1 y D2, (como se demostrará más adelante) y además porque, ahora se cita el artículo Saikia y Colaboradores, apareciendo ahora como un nuevo hecho para sustentar la supuesta carencia de altura inventiva de la invención en estudio.

En este sentido, respetuosamente nos permitimos decir que tal razonamiento no es válido ni acertado ya que el argumento indicado por el examinador de que era conocido por la persona medianamente versada en la materia técnica que el β .fosfato tricálcico era bioabsorbible y que podía ser sustituto para la hidroxiapatita citando el artículo de Saikia y colaboradores, no había sido citado durante el trámite respectivo. De hecho, en el oficio No.10258 del 18 de junio de 2013, no se indica porqué se considera que dicho artículo fuera relevante para afectar el nivel inventivo de la solicitud.

Con base en el análisis del documento D1 y D2, se ha negado la patente mediante la resolución 86133 y NO HABIA SIDO CITADO ANTERIORMENTE EL ARTÍCULO EN CUESTIÓN, tal y como se puede apreciar de la simple lectura del examen técnico del oficio 10258 del 18 de junio de 2013, dicho argumento ahora fundatorio de la resolución que se impugna, no fue considerado en dicho examen.

Es decir, se ha emitido una resolución sin que PREVIAMENTE se haya discutido este argumento en el examen técnico correspondiente, y en esta resolución 86133 del 26 de diciembre de 2013, es citado como hecho fundatorio de la falta de nivel inventivo de la presente solicitud. Lo anterior deja al solicitante en completo estado de indefensión debido a que NO fue posible ejercer el derecho de réplica correspondiente en el momento procesal oportuno, lo cual entonces vulnera lo consignado en el ARTICULO 29 de la Constitución Política de Colombia el cual establece que "EL DEBIDO PROCESO SE APLICARÁ A TODA CLASE DE ACTUACIONES JUDICIALES Y ADMINISTRATIVAS..."

Esto en relación con el artículo 45 de la Decisión 486 de la Comunidad Andina de Naciones que otorga el derecho al solicitante de contestar o manifestarse en relación a lo analizado en el examen de patentabilidad.

El artículo 29 de la Carta Política dispone que el debido proceso debe observarse en toda clase de actuaciones judiciales y administrativas, es decir que obliga, no solamente a los jueces sino también a los organismos y dependencias de la administración pública.

El debido proceso administrativo consiste en que los actos y actuaciones de las autoridades administrativas deben ajustarse no sólo al ordenamiento jurídico legal sino a los preceptos constitucionales. Se pretende garantizar el correcto ejercicio

13

21

de la administración pública a través de la expedición de actos administrativos que no resulten arbitrarios y, en consecuencia, contrarios a los principios del Estado de derecho.

Ello en virtud de que *“toda autoridad tiene sus competencias definidas dentro del ordenamiento jurídico y debe ejercer sus funciones con sujeción al principio de legalidad, a fin de que los derechos e intereses de los administrados cuenten con la garantía de defensa necesaria ante eventuales actuaciones abusivas, realizadas por fuera de los mandatos constitucionales, legales o reglamentarios vigentes”* [Cfr. Corte Constitucional. Sentencia T-1341 del 11 de diciembre de 2001 (M.P. Álvaro Tafur Galvis)].

De la aplicación del principio del debido proceso se desprende que los administrados tienen derecho a conocer las actuaciones de la administración, a pedir y a controvertir las pruebas, a ejercer con plenitud su derecho de defensa, a impugnar los actos administrativos y en fin, a gozar de todas las garantías establecidas en su beneficio, lo cual se vio vulnerado en el caso que nos ocupa, al no tener en el momento procesal oportuno los elementos con los cuales la autoridad ahora esta fundamentando la decisión 86133 la cual niega el beneficio de patente a la presente solicitud a través de la citación del documento D1 y D2 (ya antes mencionados) y de un nuevo hecho como lo es la citación del artículo de Saikia y Colaboradores.

Sobre el debido proceso administrativo la Corte se ha manifestado en reiteradas oportunidades [por ejemplo las sentencias T-442 del 3 de julio de 1992 (M.P. Simón Rodríguez Rodríguez), T-020 del 10 de febrero de 1998 (M.P. Jorge Arango Mejía), T-386 del 30 de julio de 1998 (M.P. Carlos Gaviria Díaz), T-009 del 18 de enero de 2000 (M.P. Eduardo Cifuentes Muñoz) y T-1013 del 10 de diciembre de 1999 (M.P. Alfredo Beltrán Sierra).] y ha precisado que su cobertura se extiende a todo el ejercicio que debe desarrollar la administración pública en la realización de sus objetivos y fines estatales, entre los que se encuentran por supuesto el otorgamiento del derecho de patentes.

Al respecto la Corte Constitucional se ha manifestado como sigue:

“La garantía del debido proceso, plasmada en la Constitución colombiana como derecho fundamental de aplicación inmediata (artículo 85) y consignada, entre otras, en la Declaración Universal de Derechos Humanos de 1948 (artículos 10 y 11), en la Declaración Americana de los Derechos y Deberes del Hombre proclamada el mismo año (artículo XXVI) y en la Convención Americana sobre Derechos Humanos (Pacto de San José de Costa Rica, 1969, Artículos 8 y 9), no consiste solamente en las posibilidades de defensa o en la oportunidad para interponer recursos, como parece entenderlo el juzgado de primera instancia, sino que exige, además, como lo expresa el artículo 29 de la Carta, el ajuste a las normas preexistentes al acto que se imputa; la competencia de la autoridad judicial o administrativa que orienta el proceso; la aplicación del principio de favorabilidad en materia penal; el derecho a una resolución que defina las cuestiones jurídicas planteadas sin dilaciones injustificadas; la ocasión de presentar pruebas y de controvertir las que se alleguen en contra y, desde luego, la plena observancia de las

4

formas propias de cada proceso según sus características" [Cfr. Corte Constitucional. Sentencia T-460 del 15 de julio de 1992 (M.P. José Gregorio Hernández Galindo)].

El derecho de defensa en materia administrativa, el cual ha sido vulnerado por la sentencia que recurrida, se traduce en la facultad que tiene el administrado para conocer la actuación o proceso administrativo que se le adelante e impugnar o contradecir las pruebas y las providencias que le sean adversas a sus intereses. La administración debe garantizar al ciudadano interesado tal derecho y cualquier actuación que desconozca dicha garantía es contraria a la Constitución.

No obstante lo anterior, se fundamentará lo relativo a la sentencia recurrida en su aspecto técnico en cuanto a que el señor examinador ha considerado que no se cumple con el requisito de Nivel Inventivo en la presente invención.

En este sentido, es claro que el examinador indica que una persona versada en la material podría tomar fosfato tricálcico de un compuesto X y reemplazar con este, la HA de un compuesto Y, que además contiene quitosano, reforzando este argumento con la citación del artículo de Saikia y colaboradores.

Sobre este particular, respetuosamente nos permitimos decir que el examinador menosprecia la formulación aquí reclamada, sin tener en cuenta que en química las cantidades exactas de los componentes determina su comportamiento químico, mecánico y biológico, un ejemplo muy claro lo tenemos en el caso de la HA y el fosfato tricalcio, que como se muestra a continuación los dos productos tienen la misma composición química pero en diferentes proporciones de acuerdo con lo mostrado en la siguiente tabla No 1 y tabla No 2 .

TABLA No 1. Composición química referida al porcentaje en peso de cada Elemento para HA y β -FTC

PRODUCTO	FORMULA QUIMICA	%Ca	% P	% O	%H
Hidroxiapatita	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	39,89	18,5	41,41	<u>0,2</u>
β - Fosfato tricalcico	$\text{Ca}_3(\text{PO}_4)_2$	38,76	19,97	41,26	<u>0</u>

Fuente: FRANCO, y colaboradores. Procesamiento cerámico de β -FTC para la fabricación de piezas implantables. En: boletín de la sociedadespañola de Cerámica y Vidrio. 2007, vol. 5, p. 6.

Tabla 2. Constantes del producto de solubilidad para β -FTC y HA

compuesto	FORMULA QUIMICA	Ca/P	Solubilidad 25 oC	Solubilidad 37 oC
Hidroxiapatita	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1,67	<u>58.4</u>	<u>58.6</u>
β - Fosfato tricalcico	$\text{Ca}_3(\text{PO}_4)_2$	1.5	<u>28.9</u>	<u>29.5</u>

Fuente: FERNÁNDEZ, Enrique. Obtención y caracterización de nuevos cementos óseos de fosfatos de calcio en el sistema $\text{CaHPO}_4\text{-}\alpha\text{-Ca}_3(\text{PO}_4)_2$: cementos de fosfato de calcio. Trabajo de grado ingeniero de materiales. Barcelona: PC. Departamento de ciencia de materiales e ingeniería metalúrgica, 1996. P.13.

En este sentido, el señor examinador podrá notar que la hidroxiapatita (HA), con formula química $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, tiene propiedades muy diferentes a las propiedades del fosfato, con formula $\text{Ca}_3(\text{PO}_4)_2$, si miramos la tabla No 3 , vemos que la HA , es más cristalina, menos soluble, no degradable y por lo tanto no es reemplazo adecuado para hueso nuevo.

En la siguiente tabla se hace una comparación fisicoquímicas y biológicas de la HA y el β -fosfato tricálcico en donde se puede observar que éste último es menos cristalino muy soluble, se degrada completamente y es reemplazado por hueso nuevo.

7

TABLA No 3.
Diferencias entre la Ha y el β - fosfato tricalcico

PRODUCTO	PROPIEDADES FISICAS Y QUIMICAS	TASA DE RESORCION	FORMACIÓN ÓSEA	PROPIEDADES BIOLOGICAS
Hidroxiapatita	Mas cristalina ¹ Vibración de ondas diferente para Ha y β. FTC ¹ Relación Calcio Fosforo = 1.67 ² Baja solubilidad ^{4,5} A nivel de distribución de nano partículas, esta es bimodal con picos alrededor de 40 - 500 μm, y un promedio de 275 μm ⁵ Al comparar Microtextura electrónica de partículas de Ha y β-FTC las de Ha son menos rugosas ⁵ Mayor resistencia comprensiva ⁶ En solución salina hay menor concentración de iones Ca ⁶ La fórmula química de la Ha es $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, con una composición teorica de 39.68 wt% Ca, 18.45 wt% P; Ca/P razón wt de 2.151, y razón molar Ca/P 1.667 ⁷ A un pH entre 4,2 y 8 es muy estable ⁷ (es prácticamente insoluble)	Muy lenta ^{1,6,7} Se incorpora al hueso pero no se degrada ² Estable en medio fisiológico (no se reabsorbe) ^{4,5,7}	Al no degradarse no hay neoformación ósea ³ Tasa de neoformación ósea muy lenta ⁶	Bioactiva ¹ Alta osteocompatibilidad ⁶ Menos soluble in vitro e in vivo ^{6,7} Buena biocompatibilidad ⁷
β - Fosfato tricalcico	Menos cristalino ¹ Vibración de ondas diferente para Ha y β. FTC ¹ Relación Calcio Fosforo = 1.5 ²	Rápida ^{2,7} Se incorpora al hueso y se reabsorbe ^{2,7} Se reabsorbe	Se reabsorbe completamente siendo reemplazado por hueso nuevo ^{3,7}	Bioactiva ¹ El β-FTC presenta mayor presencia de microporos y macroporos lo cual

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94

8

	Muy soluble ^{4,5} A nivel de distribución de nanopartículas, esta es multimodal con 2 picos, el primero alrededor de 400 μm, y el segundo en los 800 μm, con solapamiento de partículas entre 1000 y 1450 μm ⁵ Al comparar la micro textura electrónica de partículas de Ha y β-FTC las de β-FTC son más rugosas ⁵ Mayor resistencia compresiva ⁶ En solución salina hay mayor concentración de iones Ca ⁶ La fórmula química del β-FTC es $\text{Ca}_3(\text{PO}_4)_2$, con una relación molar de 1.5 ⁷	gradualmente ⁴ Tasa de reabsorción 10.20 veces más rápida que la Ha ⁵		permite la interacción con fluidos y mejor solubilidad ⁶ La mayor macroporosidad permite un mejor anclaje celular en el β-FTC ⁵ Alta osteocompatibilidad ⁶ Más soluble in vitro e in vivo ⁶ Buena biocompatibilidad ⁷
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En este orden de ideas, es claro que estas diferencias de comportamiento biológico de las dos sustancias, son solo explicadas por la composición exacta de cada componente, por lo que no es viable que a una persona versada en la materia se viera motivada a mezclar productos sin tener en cuenta las formulaciones indicadas. Sobre este particular, dirigimos la atención del señor examinador a la página 1304 en conclusiones del artículo titulado "Differentiation between Hydroxyapatite and β -Tricalcium phosphate by means of μ -Raman Spectroscopy de R Cuscó y Colaboradores publicado en 1998 en Journal of the European Ceramic Society 18, 1301-1305, en donde se pone de manifiesto que el estudio comparativo de la estructura de la HA y el beta-TCP proporciona un criterio adecuado para diferenciar entre estos dos compuestos de fosfato de calcio **con un comportamiento en bioactividad altamente diferente.**

Sumado a lo anterior, en el caso de un producto que contenga quitosano, como en el ejemplo del señor examinador, una mezcla sin tener en cuenta las proporciones entre dicho componente y los otros constituyentes de la composición, alteraría las propiedades deseadas y propuestas como son: dureza, moldeabilidad, tiempo de endurecimiento, etc. Más aún, una persona versada en la materia consideraría que puede ser peligroso porque al aumentar indiscriminadamente la cantidad de quitosano, **se disminuiría notablemente el pH** del biocompuesto lo que llevaría a **una muerte celular, necrosis tisular e inflamación incontrolada**, entre otras complicaciones. Es por esto, que la presente invención encontró que la relación ahora indicada entre la fase sólida y la fase líquida es importante y contribuye a que el material cerámico de la invención presente las características deseadas.

En relación con la afirmación del señor examinador en cuanto a que "...para la persona versada en la materia se tiene que el β -fosfatotricálcico es absorbible comparado con la hidroxiapatita, sea esta hidroxiapatita de origen biológico o sintética, y que el empleo de β -fosfato tricálcico promueve la formación de nuevo hueso no solo sobre los implantes sino también en los poros creados en poco tiempo sin la necesidad de tener tejido autólogo³" nos permitimos manifestar que el documento citado ahora como un nuevo hecho: "Calcium phosphate ceramics as bone graft substitutes in filling tumor defects de Saika, y Colaboradores publicado en Indian J. Orthop. 2008 Apr – Jun, 42(2). 169 – 172 que si bien dicho documento indica que el fosfato tricálcico podría ser utilizado como un posible reemplazo de la Hidroxiapatita para formar tejido óseo y por sus cualidades reabsorbibles, también es cierto que se podría reemplazar la hidroxiapatita por yeso común (sulfato de calcio) y obtendría el mismo efecto.

En este orden de ideas, es necesario hacer énfasis sobre el hecho que siempre se ha insistido en la necesidad de un material que además de ser biocompatible y bioreabsorbible, **sea capaz de estimular la neoformación en sitios de difícil o imposible cicatrización espontánea por regeneración ósea**, es decir, para **defectos de tamaño crítico**, por lo tanto, debe quedar claro que **no** es suficiente con que sean osteoconductivos (cualquier osteoconductivo como la Hidroxiapatita, el fosfato tricálcico y los sulfatos de calcio forman hueso en defectos óseos normales), sino que el reto es que el producto desarrollado **tenga**

propiedades osteopromotoras y osteoinductivas para que logren lo propuesto, y esto es precisamente lo que se ha conseguido con el biocompuesto cerámico de la presente invención. Esto se demuestra de manera clara y contundente en el artículo titulado "*Effects of calcium phosphate (CaP)/chitosan composite on bone healing in rats: Calcium phosphate induces osteon formation.*" realizado por los inventores y del cual se anexa una copia informal al presente escrito.

En relación con el artículo de Saika y colaboradores citado ahora en la resolución impugnada, es importante señalar que el mismo no es un buen ejemplo para materiales osteopromotres y osteoinductivos por varias razones:

- 1). En materiales y métodos se toma como un criterio de exclusión: "(i) Very large benign tumor, ...";
- 2) La hidroxiapatita utilizada fue de origen Bovino, por lo que no se trata de un sustituto óseo sino de un xenoinjerto que se define como el tejido proveniente de una especie diferente (hueso de origen bovino)
- 3) El fosfato tricálcico utilizado se usó solo en defectos pequeños: Small defects in the diaphyses were filled with β -TCP...
- 4) En algunos casos (seguramente en los defectos de mayor tamaño), se combinó la cerámica con hueso autologo: In eight cases autogenous cancellous bone graft harvested from the iliac crest and/ or contra lateral fibular graft was used along with the calcium phosphate material.", con lo cual, contrario a lo manifestado por el señor examinador, en dicho artículo se está utilizando entonces un autoinjerto.

Sumado a lo anterior, si se revisa la conclusión consignada en dicho artículo: "these implants are bio-compatible and osteo-conductive", es claro entonces que los sustitutos óseos de tipo fosfocálcico son solo osteconductores y que su uso está limitado a defectos pequeños. Es más, dicho documento sugiere que para un uso en defectos de tamaño grande deberá recurrirse a someter al paciente a un trauma adicional para tomar un hueso autologo, y adicionarle proteínas morfogenéticas (mediante la combinación de sangre tomada del paciente, como se menciona en el artículo). (Saika, et al. Indian J. Orthop. 2008 Apr – Jun, 42(2). 169 – 172. Disponible en: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2759621/>)

Es este sentido, es importante dirigir la atención de ese despacho sobre el hecho que el producto de la presente invención no busca obtener únicamente una osteconductividad superior, sino una osteopromoción y una osteoinducción mejoradas.

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11

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COLOMBIA

En este orden de ideas, es necesario señalar que en los documentos anexados durante el proceso se ha mostrado cómo efectivamente el biocompuesto para el que se ha solicitado la patente: es osteopromotor, estimulante de regeneración ósea en sitios de difícil cicatrización. Como se mencionó más arriba, esto se demuestra de manera clara y contundente en el artículo realizado por los inventores en el laboratorio del Dpto. de ingeniería de materiales de la universidad de Monash-Australia y del cual se anexa una copia informal al presente escrito que reporta un estudio reciente se evidencia que el biocompuesto de la invención estimula:

1. Tasa de crecimiento óseo más alta que en defectos no injertados
2. Histología similar al remodelamiento osteonico en hueso cortical.
3. Tasa de crecimiento óseo muy cercano a la tasa de degradación del material .

Estas cualidades no se observan o se anticipan en la literatura científica para este tipo de material (incluyendo los ejemplos citados por el señor examinador), y que solo se explican como resultado de la interacción sinérgica de todos los componentes del biocompuesto: fase solida fosfato tricálcico, óxido de calcio, oxido de zinc, y la fase liquida de quitosano.

Sobre este particular, es importante anotar que para la producción de las diferentes mezclas, lo primero que se realizó fue elegir la cantidad de mezclas que se iban a obtener y la cantidad de réplicas que se harían para cada mezcla, de manera que se obtuviera la máxima información, los valores que se eligieron para cada composición, fueron el producto de innumerables ensayos preliminares realizados.

En la presente solicitud se planteó que se harían todas las combinaciones posibles entre los porcentajes escogidos para cada componente a utilizar que corresponde a un peso determinado, es decir, se tienen los componentes y se escogieron los porcentajes exactos. Es decir se trabajó con la proporción exacta obtenida durante la investigación; el cálculo para las proporciones de los tres componentes se realizó mediante un diseño de modelo cuadrático, donde se muestran los contornos de la mezcla y las regiones para las cuales se obtiene los valores de pH requeridos por los cirujanos.

En este sentido, dirigimos la atención del señor examinador a las siguientes referencias bibliográficas

1. FRANCO, et al y otros y colaboradores. Procesamiento cerámico de β -FTC para la fabricación de piezas implantables. En: boletín de la sociedad española de Cerámica y Vidrio. 2007, vol. 5, p. 6.
- 11

2. R. França, et al. Nanoscale surface characterization of biphasic calcium phosphate, with comparisons to calcium hydroxyapatite and β -tricalcium phosphate bioceramics, Journal of Colloid and Interface Science (2013), doi: <http://dx.doi.org/10.1016/j.jcis.2013.12.055>
3. Rumi Fujita, et al. Bone Augmentation Osteogenesis Using Hydroxyapatite and β -Tricalcium Phosphate Blocks . J Oral Maxillofac Surg 61:1045-1053, 2003
4. S.M. Best et al. Bioceramics: Past, present and for the future. Journal of the European Ceramic Society 28 (2008) 1319–1327.

En relación con la formulación del problema técnico hecha por el examinador, cuando indica que *“...el problema técnico objetivo que pretende resolver esta invención se puede formular así: “¿cómo modificar la composición de D1 para que se realicen los procesos de bioabsorción del implante y recuperación del tejido óseo de forma más eficiente?”*, respetuosamente nos permitimos decir que es una consideración bastante desacertada y que se queda corta porque el objetivo de la presente invención va más allá. En este sentido, se dirige la atención del señor examinador a la parte de antecedentes de la invención **párrafo 20** , donde se establece que *“ , como conclusión de la revisión del estado del arte se dice : “ En este sentido, aún existe una necesidad no satisfecha en el estado de la técnica de un material cerámico que presente características fisicoquímicas adecuadas que permitan un fácil moldeo y fraguado del material con el fin de facilitar la aplicación del mismo en cavidades óseas de difícil cicatrización”*.

De hecho, durante todo el documento, se hace énfasis, en que uno de los objetivos es lograr la formación ósea en defectos de **difícil cicatrización** y se resalta, que esta sería una de la aplicaciones del producto, por ejemplo en el **Párrafo 5 (resumen)** se dice *“Esta invención se encuentra dirigida a un biocompuesto cerámico para regeneración ósea que presenta un rango de pH de 6,5 a 8,5 con una plasticidad inicial que permite su fácil moldeo “in situ” y un endurecimiento después de 7 minutos, que hace posible que permanezca en el lugar indicado mientras ocurre el proceso de cicatrización. Adicionalmente, el biocompuesto muestra capacidad de estimular la formación ósea en defectos de difícil cicatrización.”*.

Igualmente en el **párrafo 15 del campo tecnológico** se afirma que *“El biocompuesto de la invención también es útil... En cirugía ósea, en el tratamiento de las secuelas de procesos patológicos o infecciosos como los efectos causados por tumores benignos y malignos, así como la osteomielitis, los cuales generan cavidades óseas que **difícilmente cicatrizan** en forma espontánea”*.

Más adelante en el **párrafo 25** se dice que *“El biomaterial es biocompatible, osteoconductor y además, estimula la formación ósea en sitios de difícil o imposible cicatrización espontánea (defectos de “tamaño crítico”), debido a sus propiedades osteopromotoras. Estas cualidades se evidenciaron en estudios experimentales realizados en*

12

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modelos animales a los cuales se les indujo defectos de "tamaño crítico", es decir, defectos que no cicatrizarían espontáneamente en toda la vida del animal.

Es claro que para lograr regenerar un tamaño crítico de defecto es necesario garantizar la vascularización, que sirva de soporte para la neo formación ósea; el biocompuesto cerámico, por ende, va más allá de ser un simple sustituto óseo con unas adecuadas características físicas y clínicas, como se ha presentado desde el inicio del proceso, la solución que se buscaba en el campo tecnológico era lograr la regeneración de este tipo de defectos (Tamaño Crítico), en el proceso se ha suministrado suficiente evidencia de la capacidad del Biocompuesto en producir regeneración de defectos óseo de difícil o imposible cicatrización espontánea y con las últimas pruebas realizadas en el laboratorio de ingeniería de materiales de la Universidad de Monash Australia, se muestra una gran actividad angiogénica y una tasa alta de neoformación ósea, esta información esta descrita en el artículo realizado por los inventores y de pronta aparición en el Journal Tissue Engineering, y cuyo abstract, puede ser consultado en la página <http://www.ncbi.nlm.nih.gov/pubmed/24460696>.

Así las cosas, solicitamos al señor examinador considerar de nuevo el problema abordado por la presente solicitud y utilizar el acercamiento "problema-solución" con el cual será claro que la invención en estudio no es, para nada, evidente a partir de lo mostrado en la técnica anterior para una persona medianamente versada en la materia.

Cabe anotar lo que ha establecido el Tribunal de Justicia de la Comunidad Andina, Proceso 105-IP-2000 en relación al nivel inventivo:

"El invento puede constituir una derivación del estado de la técnica, pero para calificar su carácter de patentable, es preciso determinar si dicha derivación no resulta evidente, como se ha dicho, para una persona normalmente versada en la materia. Surge por consiguiente un requisito adicional al de la novedad: el nivel inventivo, del cual se desprende que la invención, además de no ser obvia para un experto medio, debe ser siempre el resultado de una actividad creativa del hombre, sin que ello signifique que para alcanzar la regla técnica propuesta, no se puedan utilizar procedimientos o métodos comunes o ya conocidos en el área técnica correspondiente. Por ello, el juicio del nivel inventivo no puede ser elaborado con criterios generales, sino que dependerá de las especiales circunstancias de cada caso". Énfasis añadido.

En cuanto al nivel inventivo, afirma el Tribunal que implica un salto cualitativo en relación con la técnica existente, presupone que la invención además de no ser obvia para una persona del oficio normalmente versada en la materia técnica, debe ser siempre el resultado de una actividad creativa del hombre, en ese sentido, la regla técnica era, hasta antes de la presente invención la utilización de hidroxiapatita en composiciones endurecibles en composiciones a base de quitosano (D1) y composiciones que contienen fosfato de calcio para reparación

de tejidos duros (D2), pero ninguno de ellos sugiere de manera clara y nada ambigua la obtención de composiciones para biocompuestos cerámicos para la regeneración ósea conteniendo proporciones específicas tanto de la fase sólida como de la fase líquida.

Por lo anterior, se puede apreciar que del arte previo aplicable a la presente solicitud NO SE EVIDENCIAN NI SE LOGRAN LAS VENTAJAS DE LA INVENCION, es decir, un Biocompuesto cerámico para regeneración ósea con aplicación en defectos de tamaño crítico.

Ahora bien, el análisis de nivel inventivo indicado en la resolución impugnada es claramente el resultado de un análisis del tipo "hindsight". De hecho, este tipo de análisis no es permitido para desvirtuar el concepto de altura inventiva.

En este tipo de análisis, el examinador no enfoca su análisis en el momento en el que el solicitante plantea un problema con el fin de solucionar fallas técnicas existentes en los productos convencionales de un campo técnico específico, sino que se enfoca en la solución dada al problema planteado y en la búsqueda de documentos con conceptos análogos. A este respecto dirijo la atención del examinador al "Manual para Examen de Solicitudes de Patentes de Invención en las Oficinas de Propiedad Industrial de los Países de la Comunidad Andina, publicado en la página Web de la Comunidad Andina, donde se establece en la página 74 en cuanto al nivel inventivo "...que la búsqueda de anterioridades se efectúa a posteriori, tomando como punto de partida la misma invención,...el examinador debe realizar el esfuerzo intelectual de colocarse en la situación que ha tenido que afrontar el técnico con conocimientos medios en la materia en un momento en que la invención no era conocida, es decir antes de la invención".(El subrayado es nuestro)

En el presente caso, para el examinador puede resultar "obvio" que el objeto de la presente solicitud se derive de la materia objeto del documento citado sin dar mayor importancia al trabajo desplegado y a la capacidad inventiva del solicitante para lograr llegar a la solución del problema planteado. Es decir, que el examinador no hace su análisis partiendo del problema mismo que existía en la técnica en el momento en que el solicitante dirige todos sus esfuerzos para encontrar una solución, sino que enfoca su estudio en el momento en que el problema ya fue resuelto.

Consideramos que el examinador no ha razonado y documentado correctamente la resolución que se impugna, por lo que se ha incumplido con los artículos 14 y 18 de la decisión 486 de la Comunidad Andina de Naciones, por lo que procede la reposición del presente trámite.

192

15

Clarke, Modet & Cº

COLOMBIA

3. Teniendo en cuenta lo dicho anteriormente, solicito a usted lo siguiente:

a) Que de conformidad con lo previsto por los artículos 3 (inciso 3); 35 (inciso 2) y 59 (inciso 2) del Código Contencioso Administrativo se consideren y se resuelvan TODOS los argumentos y TODAS las cuestiones planteadas así como las que aparezcan con motivo de este recurso.

b) Que se revoque la resolución **No. 86133 emitida el 26 de diciembre de 2013** por el Superintendente de Industria y Comercio, y se proceda a conceder el privilegio de patente de invención a la solicitud de mi mandante.

Señor Superintendente,



SILVANA MARIA LÓPEZ DÍAZ

C.C. 22468787 de Barranquilla

T.P 147463 del CSJ

notificaciones@clarkemodet.com.co

15

DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Trámite

☐ Error material

☒

Modificación a una Solicitud en trámite

☒

Patente de Invención

☐

Patente de Modelo de Utilidad

☐

Registro de Diseño Industrial

☐

Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural

☐

Persona Jurídica

☒

UNIVERSIDAD AUTÓNOMA DE OCCIDENTE

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa

Micro

☐

Pequeña

☐

Mediana

☐

Otra

☐

Documento de identificación:

☐

C.C.

☐

C.E.

☐

NIT

☐

Otro

Número

Nacionalidad/País de constitución

COLOMBIA

Dirección y domicilio del titular

Calle 25 No. 115 - 85, Cali Valle

Ciudad

CALI

Dirección electrónica

No. Fax

Número telefónico

☐

Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

SILVANA MARIA LÓPEZ DÍAZ

Apellidos y Nombre

C.C. 22.468.787

Documento de identificación

147463 CSJ

Tarjeta profesional

Dirección y domicilio		Ciudad
Carrera 11 No. 86-53, Piso 6, Bogotá D.C., Colombia		Bogotá D.C.
Dirección electrónica	No. Fax	Número telefónico
notificaciones@clarkemodet.com.co	6350824	6181088

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sirvase indicar el número de su radicación o protocolo de poder general

4. Datos del trámite

Corrección:

Expediente No. 11-037.516

Aparece _____

Debe ser _____

Modificación:

Presentación Solicitud Divisional.

Modificación al capítulo descriptivo.
☐ _____

☒ Modificación al capítulo Reivindicatorio
Presento junto con este memorial nuevo capítulo nuevo descriptivo que consta de 3 reivindicaciones.

Modificación al resumen.
☐ _____

Modificación a los dibujos.
☐ _____

Modificación al solicitante.

5. Anexos

☒ Comprobante de pago de la tasa para la presentación de la corrección y/o modificación

☐ Poder, si fuere el caso.

☒ Documento que contiene las modificaciones o correcciones.

☐ Copia de la solicitud y sus anexos en formato magnético.

6. Firma

SILVANA MARIA LÓPEZ DÍAZ

Nombre y apellidos

C.C. 22.468.787

Firma

Tarjeta Profesional T.P. 147463 (CS)

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

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Industria y Comercio
SUPERINTENDENCIA

RECIBO DE CAJA

No. 14 - 0010903

Bogotá D.C., Enero 31 de 2014 - 09:29:37

RECIBO DE CLASIFICACION DET

NI 830.008.643

RE

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
RECIBO DE CA	999999999	299	03/01/2014		546.000.00
RECIBO DE CA	999999999	300	03/01/2014		546.000.00
RECIBO DE CA	999999999	1070	08/01/2014		546.000.00
RECIBO DE CA	999999999	1071	08/01/2014		546.000.00
RECIBO DE CA	9999999	1072	08/01/2014		546.000.00
RECIBO DE CA	9999999	1073	08/01/2014		546.000.00
RECIBO DE CA	999999999	1116	08/01/2014		31.000.00
RECIBO DE CA	999999999	1117	08/01/2014		31.000.00
RECIBO DE CA	999999999	1133	08/01/2014		106.000.00

*** Conceptos Pagados ***

CANT. RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
150.057.000	CAMBIO DE FORMALIDAD Y MODIFICACIONES EN CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA	128.000.00	128.000.00
			\$128.000.00

SON: CIENTO VEINTIOCHIL MIL PESOS MONEDA CORRIENTE***

Responsable:

Recibo de Caja Aplicado a Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 11-037516- -00011-0000

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105
16
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ANEXOS

1. Nuevo capítulo reivindicatorio
2. Artículo titulado "Differentiation between Hydroxyapatite and β -Tricalcium phosphate by means of μ -Raman Spectroscopy de R Cuscó y Colaboradores publicado en 1998 en Journal of the European Ceramic Society 18, 1301-1305, en donde se pone de manifiesto que el estudio comparativo de la estructura de la HA y el beta-TCP proporciona un criterio adecuado para diferenciar entre estos dos compuestos de fosfato de calcio con un comportamiento en bioactividad altamente diferente
3. Artículo titulado "*Effects of calcium phosphate (CaP)/chitosan composite on bone healing in rats: Calcium phosphate induces osteon formation.*" realizado por los inventores de la presente invención.

REIVINDICACIONES

1. Un biocompuesto cerámico para re-generación ósea que estimula la formación y el aumento
5 del volumen óseo, caracterizado porque comprende:

a) una fase sólida que comprende:

- i) entre 92% a 96% de β -fosfato tricálcico en relación con el peso de la fase
sólida total;
- ii) entre 2.5% a 6.5% de óxido de calcio en relación con el peso total de la
10 fase sólida; y
- iii) entre 0.5% a 3.5% de óxido de zinc en relación con el peso total de la
fase sólida; y

b) una fase líquida que comprende quitosano;

caracterizado además porque la relación fase sólida a fase líquida es de 0.67.

15

2. Un biomaterial cerámico caracterizado porque comprende un biocompuesto de conformidad
con la reivindicación 1 en combinación con un injerto seleccionado del grupo que consiste de
autoinjertos, homoinjertos, xenoinjertos y aloplásticos.

20

3. Un biomaterial cerámico caracterizado porque comprende un biocompuesto de conformidad
con la reivindicación 1 ó 2 en combinación con sustancias osteopromotoras tal como colágeno,
aspirado de médula ósea y plasma rico en plaquetas o con sustancias osteoinductores tal como
proteínas morfogenéticas.

25

Differentiation between Hydroxyapatite and β -Tricalcium Phosphate by Means of μ -Raman Spectroscopy

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^bInstituto de Cerámica, University of Santiago de Compostela, 15706 Santiago de Compostela, Spain

^cInstituto de Cerámica y Vidrio, Consejo Superior de Investigaciones Científicas (CSIC), Arganda del Rey, Madrid, Spain

Abstract

We present a comparative study of the Raman spectra of polycrystalline β -tricalcium phosphate (β -TCP) and hydroxyapatite (HA) over their whole optical frequency range. Due to their structural differences, Raman scattering permits to differentiate between these two calcium phosphates, not only through the detection of vibrational modes associated with the OH^- group of hydroxyapatite, but also by comparing the bands arising from internal PO_4^{3-} modes. The widths of the Raman-scattering bands associated with PO_4^{3-} bond-bending modes and the frequency gap between them are characteristic features that can be used to distinguish between β -TCP and HA. The method is used to identify a HA layer a few microns thick which precipitates on the surface of TCP–wollastonite eutectic soaked in simulated body fluid. © 1998 Elsevier Science Limited. All rights reserved

1 Introduction

Hydroxyapatite (HA) is a calcium phosphate compound which plays a central role in the development of materials for cementless prosthetic implants. Because of its bioactivity properties, HA joins directly to the bone and favours implant fixation, whereas, by contrast, tricalcium phosphate (TCP), another closely related calcium phosphate compound whose stable phase at room temperature is β -TCP, is resorbable.^{1,2}

Since during the synthesis of HA variable quantities of β -TCP can be produced depending on pH conditions, Ca/P ratio, ageing time and temperature, the characterization of the final product to

check for the presence of β -TCP is of great importance. In fact, the variability of results found in the literature regarding the actual bonding of HA-based implants with bone tissue might be partly due to the presence of a certain amount of β -TCP in the synthetic HA. For other implant materials such as bioglasses it has been recognized that the formation of a HA layer on the surface of the implant³ is correlated with the bioactivity index of the material. Micro-Raman spectroscopy is a powerful and nondestructive tool that can be used to identify locally different phases present in the material. In particular it can be used to analyse, on a length scale of the order of $1\text{ }\mu\text{m}$, the chemical and structural changes occurring on the surface of bioactive materials exposed to simulated body fluid (SBF).

2 Experimental

To clearly establish the differences between the Raman spectra of HA and β -TCP, we recorded Raman spectra of pure polycrystalline samples of each compound obtained by chemical synthesis. Pure polycrystalline HA was obtained at room temperature by stoichiometric reaction of $\text{Ca}(\text{NO}_3)_2$ 0.431 M and H_3PO_4 0.258 M solutions at pH 11 in a closed reactor with continuous stirring, leaving the precipitate to age for 1 day, also with continuous stirring. After drying at 110°C for 24 h, the precipitate was isostatically pressed into pellets, which were subsequently heated in air atmosphere in an electric furnace at a rate of $5^\circ\text{C}/\text{min}$ up to 800°C . The samples were maintained at this temperature for 1 h, with a water vapour pressure of 10 mm Hg, before being furnace cooled. The Ca/P molar ratio of these samples was determined by ICP-AES and found to be 1.67 ± 0.01 . X-ray

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diffraction analysis only revealed the presence of the HA phase. Pure polycrystalline β -TCP samples were obtained from $\text{Ca}(\text{NO}_3)_2$ 0.7 M and H_3PO_4 0.5 M solutions at pH 11 with an initial mixing molar ratio $\text{Ca}/\text{P}=1.40$ in order to avoid the formation of small quantities of HA. The precipitate was dried at 110°C for 24 h and isostatically pressed into pellets. These were heated in an electric furnace at a rate of $5^\circ\text{C}/\text{min}$ up to 1000°C , and annealed at this temperature for 1 h. The Ca/P molar ratio of the obtained samples was determined by ICP-AES and was found to be 1.50 ± 0.01 . X-ray diffraction analysis of the samples revealed a pure β -TCP phase with no detectable presence of any other calcium phosphate phase.

The calcium phosphate layer that we have characterized by means of μ -Raman spectroscopy was formed on a wollastonite-TCP bioceramic soaked in SBF at 36.5°C for 3 weeks. The wollastonite-TCP bioceramic (Bioeutectic®) was obtained by heating the eutectic composition (60% in weight wollastonite/40% in weight TCP) at 1500°C for 2 h, and by subsequently cooling the melt to 1390°C at a rate of $0.5^\circ\text{C}/\text{h}$.⁴ Eutectic microstructures composed of spherical colonies of alternating radial lamellae of wollastonite and TCP were obtained.

The Raman scattering measurements were performed using a Jobin-Yvon T64000 spectrometer equipped with a microscope. The Raman signal was detected by a multichannel CCD detector cooled with liquid nitrogen. The Raman spectra were recorded using the double subtractive configuration of the spectrometer with $100\text{-}\mu\text{m}$ slits. The light was collected in backscattering geometry through an objective of numerical aperture 0.95. The 488-nm line of an Ar^+ laser was used as excitation, focused to a spot of $\sim 1\text{ }\mu\text{m}$ diameter, with an incident power on the sample of $\sim 2\text{ mW}$.

3 Results and Discussion

The vibrational spectra of both β -TCP⁵ and HA^{6,7} exhibit a strong molecular character associated with the internal modes of the PO_4^{3-} tetrahedra.⁸

The vibrational normal modes of the free tetrahedron (T_d point group symmetry) are well-known,⁹ and give rise to four different frequencies: ν_1 , ν_2 , ν_3 and ν_4 . The ν_1 frequency corresponds to the symmetric stretching of the P-O bonds (A_1 symmetry), whereas the ν_3 frequency arises from the triply degenerate T_2 mode involving asymmetric P-O stretching and also P motion. The ν_2 frequency corresponds to the doubly degenerate O-P-O bending modes (E symmetry), and the ν_4

frequency is due to the triply degenerate T_2 modes of mainly O-P-O bending character. The values of the normal-mode frequencies of the PO_4^{3-} tetrahedron, obtained from Raman scattering measurements on phosphates in aqueous solution, are $\nu_1 = 938\text{ cm}^{-1}$, $\nu_2 = 420\text{ cm}^{-1}$, $\nu_3 = 1017\text{ cm}^{-1}$ and $\nu_4 = 567\text{ cm}^{-1}$.¹⁰ In molecular crystals, such as HA and TCP, the crystalline field induces distortions in the PO_4^{3-} tetrahedra, which change the intratehedral bond lengths and angles, and give rise to shifts and splittings of the PO_4^{3-} normal modes. Consequently, the resulting internal PO_4^{3-} bands depend on the crystallographic structure of each compound.

Figure 1 shows the Raman spectrum of HA over its whole optical frequency range. Four Raman-scattering bands, which can be straightforwardly associated with the four normal mode frequencies of the PO_4^{3-} tetrahedron mentioned above, clearly dominate the spectrum. Whereas a single, intense peak arising from the nondegenerate ν_1 mode of the PO_4^{3-} tetrahedron is detected at 962 cm^{-1} , several peaks can be observed in the bands originating from the doubly degenerate ν_2 and triply degenerate ν_4 and ν_3 tetrahedron modes, which span a frequency range of $400\text{--}490$, $570\text{--}625$ and $1020\text{--}1095\text{ cm}^{-1}$, respectively. These internal PO_4^{3-} bands are centred at frequencies between 20 and 25 cm^{-1} higher than those of the corresponding free-tetrahedron normal modes. In addition to the internal PO_4^{3-} bands, several peaks are detected between 50 and 320 cm^{-1} , which are due to translational modes of the Ca^{2+} and PO_4^{3-} sublattices

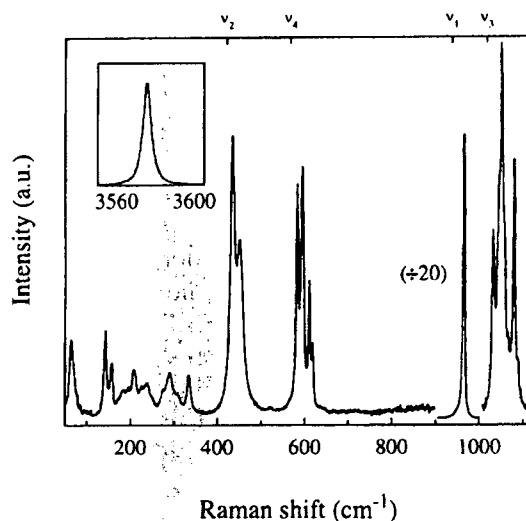


Fig. 1. Raman spectrum of pure polycrystalline HA. The frequencies of the normal modes of the free PO_4^{3-} tetrahedron are indicated by ν_1 , ν_2 , ν_3 and ν_4 on the top frequency axis. Note the different intensity scale for the ν_1 peak. The spectral region corresponding to the O-H stretching vibrations is shown in the inset. The intensity ratio between the O-H stretching peak at 3576 cm^{-1} and the symmetric stretching peak at 962 cm^{-1} is 0.22.

and rotational modes of the PO_4^{3-} group.^{6,11} The bond-stretching mode associated with the OH^- group is also detected as a sharp peak at 3576cm^{-1} , whereas the translational modes associated with the OH^- sublattice yield a Raman peak at 335cm^{-1} .^{6,7} The fact that the vibrational frequency of the OH^- bond-stretching mode is far from all the internal and external mode frequencies of the calcium phosphate compounds allows one to distinguish unambiguously between HA and TCP when this mode can be detected. However, as we shall see below, the detection of this peak above the background noise is very difficult in samples of a lesser degree of crystallinity. Therefore, to establish the distinction between HA and β -TCP one should look for differential features in the dominant PO_4^{3-} internal bands.

Figure 2 shows the Raman spectrum of β -TCP. As in the case of HA, the spectrum is dominated by the internal PO_4^{3-} bands, which are also centred around frequencies $20\text{--}30\text{cm}^{-1}$ higher than the corresponding normal modes of the PO_4^{3-} tetrahedron, indicating a similarly strong crystalline field in both structures. However, whereas β -TCP crystallizes in the $R3c$ space group and its unit cell contains 42 PO_4^{3-} tetrahedra,¹² HA crystallizes in the $P6_3/m$ space group and its unit cell contains only six PO_4^{3-} tetrahedra.¹³ As discussed in detail in Ref. 7, sizeable differences between the internal PO_4^{3-} modes in HA and β -TCP arise from the highly different degree of complexity of their respective crystalline structures. Since the number of PO_4^{3-} tetrahedra in the unit cell is seven times larger for β -TCP than for HA, a correspondingly larger number of internal modes, whose frequencies will be slightly shifted due to correlation

effects, is expected in β -TCP. Furthermore, whereas the six PO_4^{3-} tetrahedra in the HA unit cell are crystallographically equivalent, three nonequivalent types of tetrahedra can be distinguished in the β -TCP unit cell.¹² As a consequence of the differences in intratetrahedral bond lengths and angles, the spread of frequencies of the split PO_4^{3-} modes is significantly larger for β -TCP. The effects of tetrahedra distortions on the frequency regions spanned by the internal PO_4^{3-} bands have been described by an elastic model, which reproduces the trends observed in the Raman spectra taking into account the changes in the force constants due to variations in bond lengths.⁷ The effects of the existence of three nonequivalent tetrahedra which are differently distorted can be clearly observed in the internal band arising from the nondegenerate ν_1 symmetric stretching mode of the tetrahedron. Whereas a single intense peak at $\nu=962\text{cm}^{-1}$ is detected in HA, two peaks and a shoulder can be observed in β -TCP. The Raman spectra of β -TCP and HA also exhibit clear differences in the asymmetric stretching and O-P-O bond-bending regions. The bands arising from the doubly degenerate ν_2 and triply degenerate ν_4 modes, although being centred around the same frequencies, span a different frequency range in HA and β -TCP. Thus, for β -TCP we find ν_2 modes in the $370\text{--}505\text{cm}^{-1}$ range and ν_4 modes in the $530\text{--}645\text{cm}^{-1}$. Therefore, whereas Raman scattering bands arising from ν_2 and ν_4 modes are separated by a frequency gap of $\sim 120\text{cm}^{-1}$ in HA, the gap is of only $\sim 55\text{cm}^{-1}$ in β -TCP. This is a characteristic spectral feature, which can be used to distinguish between HA and β -TCP even in samples with poor crystallinity where the resolution of individual peaks in these bands as well as the detection of the O-H stretching mode of HA can be difficult. In samples with a high degree of crystallinity there are also noticeable differences at frequencies below the ν_2 band, in the lattice-mode region corresponding to Ca^{2+} and PO_4^{3-} translational modes, and PO_4^{3-} librational modes. Whereas several sharp peaks are resolved in HA, only a broad peak and a featureless band is observed in β -TCP. This is also related to the much more complex crystal structure of β -TCP, in which cation sites with partial occupancy and positional disorder of certain PO_4^{3-} groups are present.¹²

Once the Raman spectra of pure, polycrystalline reference specimens of HA and β -TCP have been characterized, μ -Raman spectroscopy can be used to distinguish between these two compounds in the calcium phosphate layers which precipitate on the surface of bioceramic materials as a result of ionic interchange with SBF. In particular, we use this technique to check that the layer of a few microns

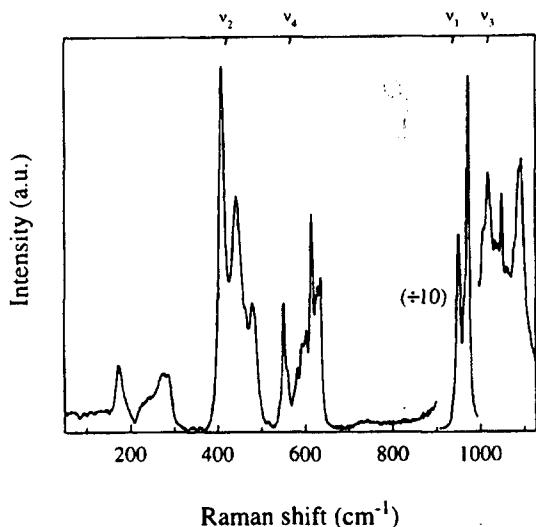


Fig. 2. Raman spectrum of pure polycrystalline β -TCP. The frequencies of the normal modes of the free PO_4^{3-} tetrahedron are indicated by ν_1 , ν_2 , ν_3 and ν_4 on the top frequency axis. Note the different intensity scale for the ν_1 band.

that precipitates on the surface of a wollastonite-TCP bioceramic soaked in SBF for several weeks is composed of HA microcrystals. In Fig. 3 we show the Raman spectrum obtained from the surface layer of the wollastonite-TCP sample described in Section 2. In the frequency range up to 1100 cm^{-1} , only the Raman scattering bands associated with the internal modes of the PO_4^{3-} tetrahedra are detected. In the ν_1 region, an intense single peak is observed at 962 cm^{-1} which, according to the previous discussion, suggests that formation of HA crystals may have taken place. However, the ν_1 peak is noticeably broader in relation to the ν_1 peak of the reference HA spectrum shown in Fig. 1. Therefore, the possibility that this peak might contain the unresolved ν_1 peaks of β -TCP (see Fig. 2) cannot be ruled out from the onset. The fact that the ν_1 peak is broader for the HA layer precipitated on wollastonite-TCP than for the reference HA sample reflects the poorer crystallinity of the former. This is not surprising considering that the precipitation conditions of synthetic HA were optimized and that the sample was subsequently heated up to 800°C to improve its crystallinity. Thus, the differences between the bond-bending internal bands of HA and β -TCP may provide a more useful criterion to discern between these two calcium phosphates in poor crystallinity samples. To confirm the identification of HA, we observe from Fig. 3 that the bands corresponding to the ν_2 and ν_4 bond-bending modes of the tetrahedra are relatively narrow and well-separated. The widths of these bands are 47 cm^{-1} for the ν_2 band and 50 cm^{-1} for the ν_4 band. These values are in better

agreement with the widths found for the HA reference sample than with those of β -TCP. Furthermore, the separation between these two bands is found to be about 110 cm^{-1} , a value close to that of $\sim 120\text{ cm}^{-1}$ found for the HA reference spectrum of Fig. 1 and clearly inconsistent with the band separation of 55 cm^{-1} found in the β -TCP reference spectrum. To further support the identification of the surface layer as HA we recorded the spectrum in the O-H stretching-mode region, which is shown in the inset of Fig. 3. A very weak signal detected around 3580 cm^{-1} confirms the presence of the OH^- group in the structure. Therefore, we conclude that HA crystals have been formed on the wollastonite-TCP bioceramic after being soaked in SBF under the conditions reported in Section 2.

4 Conclusions

We have presented a comparative study of the Raman spectra of synthetic HA and β -TCP aimed at providing suitable criteria to differentiate between these two calcium phosphate compounds with highly different bioactivity behaviour. We have shown that, apart from the modes associated with the OH^- group of HA, which are usually difficult to detect in samples with poor crystallinity, and the different number of ν_1 peaks detected in each compound, the ν_2 and ν_4 bending modes exhibit noticeable differences which can be used to discern between HA and β -TCP. In particular, the frequency gap between the bands arising from the ν_2 and ν_4 bond-bending modes of the PO_4^{3-} tetrahedra can be used as a fingerprint to distinguish between these two calcium phosphates. Therefore, Raman spectroscopy has proven to be a powerful nondestructive technique to distinguish between HA and β -TCP crystals. Finally, it has been shown by means of μ -Raman spectroscopy that HA crystals are formed on the surface of wollastonite-TCP bioceramics soaked in SBF.

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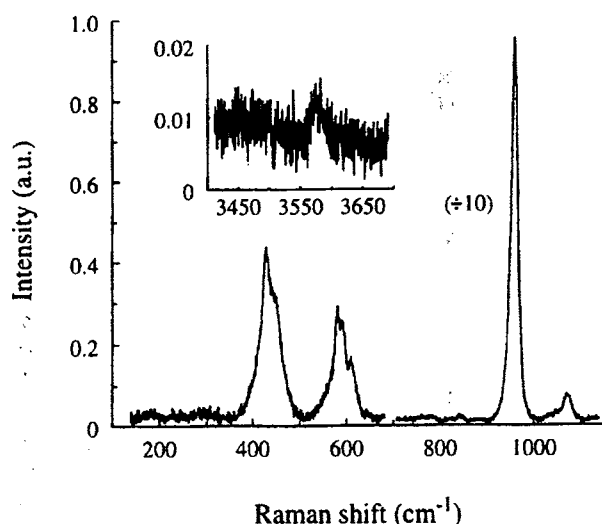


Fig. 3. Raman spectrum obtained from the surface region of a cross section of a wollastonite-TCP bioceramic soaked in SBF. Comparison with the spectrum of Fig. 1 allows us to identify this calcium phosphate layer as HA. The O-H bond stretching region of the spectra is shown in the inset. Note the different intensity scales.

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Effects of calcium phosphate (CaP)/chitosan composite on bone healing in rats: Calcium phosphate induces osteon formation

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Abstract

Vascularisation of an artificial graft represents one of the most significant challenges facing the field of bone tissue engineering. Over the past decade, strategies to vascularise artificial scaffolds have been intensively evaluated using osteoinductive calcium phosphate (CaP) biomaterials in animal models. In this work, we observed that CaP-based biomaterials implanted into rat calvarial defects showed remarkably accelerated formation and mineralization of new woven bone in defects in the initial stages, at a rate of $\sim 60 \mu\text{m/day}$ (0.8 mg/day), which was considerably higher than normal bone growth rates (several $\mu\text{m/day}$, 0.1 mg/day) in implant-free controls of the same age. Surprisingly, we also observed histological evidence of primary osteon formation, indicated by blood vessels in early-region fibrous tissue, which was encapsulated by lamellar osteocyte structures. These were later fully replaced by compact bone, indicating complete regeneration of calvarial bone. Thus, the CaP biomaterial used here is not only osteoinductive, but vasculogenic, and may have contributed to the bone regeneration, despite an absence of osteons in normal rat calvaria. Further investigation will involve how this strategy can regulate formation of vascularised cortical bone such as by control of degradation rate, and use of models of long, dense bones, to more closely approximate repair of human cortical bone.

Keywords: Calcium phosphate, Haversian remodelling, degradation rate, bone growth rate, rat calvaria

1. Introduction

Bone repair is a subject of intensive investigation in orthopaedic reconstruction because of the great clinical need for effective approaches to enhance or direct bone healing. Current approaches in bone reconstructive surgery are dominated by autografts and allografts. However, biological bone grafts all have shortcomings, such as donor site shortage and morbidity in autografting, immune rejection and the transmission of diseases (such as HIV and hepatitis virus) in allografting and cross-contamination of animal viruses associated with xenografting (1). Over the past decade, scaffold-based tissue engineering strategies have been investigated using various synthetic materials, including hydroxyapatite (HA), calcium phosphates (CaP), polyesters, chitosan and their composites (2). Among these artificial bone matrices, CaP, because of its intriguing osteoinductivity (3, 4), has been extensively evaluated *in vivo* (5, 6). Among all animal models, rats and mice are the most widely used, primarily because of their relatively low cost compared to livestock and non-human primates. However, rat and mouse bones do not have Haversian canals (7), which are the essential structures carrying the vascular network in human cortical bone. This difference between rodent and human bone structure is significant, bearing in mind that vascularisation of bone tissue engineering scaffolds has been recognised as the major obstacle to successfully achieving clinically viable artificial scaffolds (8). Therefore, the primary objective of this work is to address two questions: firstly, whether CaP implantation can result in formation of Haversian canals in rodents and, secondly, whether a rodent model can be used in evaluating bone engineering strategies, especially those aimed at promoting vascularisation of cortical bone.

Osteoinduction is a process that results in heterotopic or ectopic bone formation *in vivo*. Numerous growth factors (notably bone morphogenetic proteins; BMPs) and biomaterials such as those containing CaP display an ability to stimulate osteoinduction (9). The reported osteoinductivity of CaP is influenced by a number of physico-chemical properties of the biomaterial, such as particle size (10), surface area, crystallinity, porosity, and composition (11, 12). Particles of sizes ranging from 80 to 300 μm in diameter can induce ectopic bone formation, whereas particles greater 500 μm are not osteoinductive (13). *In vivo* studies also indicate that a specific surface area above a threshold level of 1.0 m^2/g is critical for CaP to exhibit osteoinduction (6). Moreover, biphasic CaP (mixtures of HA with CaP) generally demonstrate simultaneously increased solubility and osteoinductivity (14), whereas pure HA or amorphous tricalcium phosphate (TCP) display no osteoinductivity because of either having too high stability or too high a dissolution rate (15). Based on these considerations, it is likely that the osteoinductivity of CaP-based biomaterials is in essence controlled by their degradation kinetics, and other properties (e.g., particle size, surface area, crystallinity, porosity, and composition) influence the degradation rate and thus the osteoinductivity. Hence, a secondary objective of this work is to investigate whether the degradation rate of CaP-based particles correlates with the bone growth rate induced by such materials.

Chitosan is another biomaterial being intensively investigated for its ability to enhance osteoinductivity of CaP-based materials. Chitosan is a polysaccharide composed of glucosamine and N-acetyl glucosamine and is naturally cationic, so it can produce electrostatic unions with the glycosaminoglycan anions, proteoglycans and other negative charged molecules available in the extracellular matrix. This property has a positive influence on the bone healing process as

cytokines and growing factors are attached to glycosaminoglycans such as heparin and heparin sulphate. Chitosan is reported to be able to enhance bone healing through promoting polymorphonuclear infiltration at the healing site and their ability to bind anionic molecules such as growth factors and DNA (16). Thus, a graft of chitosan-glycosaminoglycan may help increase the concentration of growth factors released by colonising cells. For these reasons, the biomaterial used in this study is a composite of β -tricalcium phosphate (β -TCP) and chitosan in its acetylated form.

In principle, the application of biomaterials as artificial bone substitutes is aimed at healing of large bone defects that cannot heal themselves and requires a relatively large bone graft. A critical-sized defect (CSD) is defined to be the minimal defect that would not heal, regardless of how much time it is given to heal (17, 18). Clinically, the term CSD is given to a defect that has not healed within eight months of injury (19). Various animal models of bone repair with the anatomical capacity to regenerate a CSD have been developed for biomaterials research (20, 21), but the 5-mm rat calvarial bone defect is one of the most frequently used models in *in vivo* studies (22-24). Very recently, a systematic review on CSDs of the calvarial model (25) indicated that only 1.6 % of such 5.0-mm defects heal completely with newly formed bone. Therefore, we employed this model of an intramembranous bone healing process to investigate the limit of osteoinductive capacity of a CaP/chitosan composite, as well as the suitability of this rat model for the evaluation of bone tissue engineering strategies. We were especially interested to see if osteoinduction involved the specific formation of osteons or similar kinds of histological evidence of cortical bone remodelling.

116

29

2. Materials and Methods

2.1. Biomaterial preparation

The biomaterial used in this work was a paste made from solid bioceramic powder and an aqueous chitosan solution (2 wt %, pH = 4.5), which was purchased from Polimar Cienciae Nutricao S.A. The bioceramic powder was a mixture of β -TCP (Emprove[®]), calcium oxide and zinc oxide, all purchased from Merck[®]. The particle size of each powder was measured by laser granulometry using a Mastersizer200 (Malvern Instruments). The analysis was conducted using a laser diffraction liquid method on the following suspensions: β -TCP dispersed in propenol and CaO and ZnO dispersed in water. The particle diameters of the β -TCP, CaO and ZnO powders were 15, 3 and 9 μ m, respectively. CaO was incorporated mainly for adjustment of pH of the composite, and ZnO was doped for its ability to induce vascularisation at early stages, as indicated by increased markers of osteoblast differentiation, matrix maturation and bone mineralisation in a previous work (26).

A series of composites (100 samples) was systematically prepared from bioceramic powders of different β -TCP : CaO : ZnO ratios and mixed with the liquid chitosan solution at various solid/(solid + liquid) percentages. The pH values of these composites were measured. The composites (20 samples) with a pH value between 6.5 and 8.5 were considered to be safe for biological environments, and the rest (80 samples) were discarded because of anticipated toxicity. Therefore, the ratio β -TCP : CaO : ZnO : chitosan of 38.4, 1.0, 0.6 and 60 wt %, which had a pH value of $\sim$ 7.5, was thus used in the animal study.

The composite mixture was prepared as follows. The three weighted ceramic powders were gently mechanically mixed for ~5 minutes, and then the mixture was dried in a microwave oven for ~20 min. Each of these dried mixtures was added to the chitosan solution according to the designed percentage to produce a paste. The compressive strength of the paste was ~0.5 MPa. The crystallinity of β -TCP was approximately 30 %, as provided by the supplier. The CaO and ZnO powders were amorphous.

2.2. Animal model

Twelve male Wistar rats, which were 4-months old and weighed 300g on average, were used. The rats were randomly divided into three groups of 4 rats, with the groups to be examined respectively at 20-, 40-, and 60-day time points. Two bone defects (5 mm in diameter and full depth, which was ~0.8 mm) were created in the rat skulls (control and experimental sites) (Fig. 1a). The experimental protocols and the animal care was approved and supervised by the Animal Ethics Committee of the University of Valle (Cali, Colombia) and the University Autónoma de Occidente (Cali, Colombia).

2.3. Surgical procedure

Operations were performed on the rats using general anaesthesia, i.e., ketamine (50 mg/ml; 0.7 mg/kg), xilacin (2 %, 0.6 mg/kg), and acepromazinemaleate (10 mg/ml, 0.6 mg/kg). Two circular bone defects were introduced using a trephine bur with a dental implant surgical hand piece (400 rpm). After washing the rat skull with a physiological saline solution, the right bone skull defect was filled with the chitosan/ceramic paste, and the left one was left empty as a control (Fig. 1b). The three groups of rats were sacrificed separately at 20, 40 and 60 days post-implantation. To

minimise the experimental differences between the rats, the same experienced surgeon performed all the operations.

2.4. Histological and electron microscopy sample preparation

The experimental bone defects were retrieved and fixed in 2.5% glutaraldehyde Tris buffered saline (TBS) (0.01M, pH 7.4) solution over 48 hours at 4°C. After washing with TBS (0.01M, pH 7.4), control samples and two samples of each experimental group were decalcified in 15 % ethylenediaminetetraacetic acid (EDTA) water solution (pH 7.3) at 4°C for 2 months, and the other two samples of each experimental group remained undecalcified. After rinsing in TBS (0.01M, pH 7.4), the bulk decalcified and undecalcified samples were dehydrated in increasing ethanol concentrations (70-100 %) and embedded in paraffin wax for optical microscope histology or poly(methylmethacrylate) (PMMA) for electron microscopy. Then, 5-µm thick slides for histology and scanning electron microscopy (SEM) or 50-70 nm for transmission electron microscopy (TEM) were sectioned along the coronal plane (Fig. 1b) in cooling water with a microtome and an ultrathin microtome, respectively. Histological slides of decalcified bone were stained with haematoxylin-eosin (H&E), whereas those of undecalcified bone was stained with Goldner's trichrome. H&E staining discriminates biomaterial particles, which stain purple or dark red; fibrous tissue, which stains light pink, and decalcified bone, which stains orange or red. Goldner's trichrome staining discriminates immature woven bone (red) and mature lamellar bone (green/blue) in undecalcified samples. Goldner's trichrome stain is more sensitive to the level of mineralisation in undecalcified samples, discriminating regions of a high or low mineral concentration, which stain green/blue or red, respectively.

2.5. Histomorphometric analysis

The above stained samples were imaged with an Aperio ScanCope® Turbo scanner (Aperio Technologies, Vista, CA, USA/Serial Number AT1681). All scans were conducted at the same resolution and magnification (i.e., 0.497 microns per pixel, 20×). The images of the region of interest (ROI) were then processed with Adobe Photoshop® version CS2 (9.0) to obtain the masks. In this process, the selected features were designated in black, and the rest of the examined area was designated in white, and thus, a black-and-white image (i.e., mask) was created. ImageJ® 1.46r software was used to measure the area percentage of the selected features in the examination field (27).

2.9. SEM and TEM examination

The SEM samples were examined using a JEOL7001 field emission gun SEM at 20 kV. Back-scattered electron diffraction (BSED) mode was used for elemental analysis. Energy dispersive x-ray (EDX) analysis was performed at 20 kV. The TEM foils were examined with a Tecnai 20 microscope at 100-200 kV, depending on the materials present in the areas of interest.

2.10. Statistical analysis

All experiments were performed with at least six samples per animal and 4 animals per experimental group, and the statistical outputs are shown in the form of a mean with standard error ($\pm$ SE). A one-way analysis of variance (ANOVA) with Turkey's *post hoc* test was performed to analyse the significant differences, and the significance levels were set at a *p*-value of less than 0.05.

3. Results

3.1. Histology and histomorphometric analysis

Analysis of undecalcified samples: Trichrome stains

All surgical procedures were performed without complications. Histological examination (Goldner's trichrome) revealed that the control defects remained empty, with little new bone formed up to 40 days post-implantation (**Fig. 2a and b**), and the amount of new bone was considerable (~ 20 Ar. % of the defects) only in the samples of the 60-day treatment group (**Fig. 2c**). These results are in agreement with previous work on bilateral calvarial defects that are 5.0 mm in diameter, which reported that the area percentage of new bone formed in the defects was 20.24 and 22.65 % at 2 and 3 months, respectively (25).

In contrast, the formation of new bone proceeded in all the experimental sites implanted with the CaP-based biomaterial, with defects being filled with newly formed soft and/or hard tissue (**Fig. 3**). Undecalcified sections of the 20- and 40-day bone defect tissue samples stained predominately red and green by Goldner's trichome (**Fig. 3a**). The three regions (I, II and III) were classified according to their histological characterisation at high magnification, as shown in **Fig. 4**. Region I was occupied by a mixture of biomaterial particles and fibrous tissue, with the former being predominant in the 20-day tissue samples (**Fig. 4a**), with fibrous tissue and blood vessels predominant in the 40-day samples (**Fig. 4b**). In areas designated as Region I of the 40-day tissue samples, a high population of cells (fibroblasts) was observed in the brown area, with the nuclei stained dark brown (**Fig. 4b**). Region II was dominated by newly formed bone characterised by a high population of irregular-shaped cavities (**Fig. 4c**). Region III (red) was avascular cortical bone, characterised by canaliculi, with few irregular-shaped cavities (**Fig. 4d**). Almost no biomaterial particles could be observed in Region II and, especially Region III under

optical microscopy. The tissues of Regions I and II in the 40- and 60-day samples were actually located above the original level of the skin and thus were soft and hard calli. The callus would eventually be corrected on its own, and the normal bone contour would be restored(28).

It should be mentioned that the green (blue) and red stains in the present samples must be interpreted with a caution because of the grafted CaP material. Goldner's trichrome is sensitive to the level of mineralisation in bone and thus is used to discriminate newly formed relatively mature bone. With this staining method, non-mineralised (immature) bone stains red, and mineralised (mature) bone stains green/blue (29). The samples of the CaP-grafted group in the present study, however, stained in the opposite manner. The newly formed woven bone (**Fig. 4c**), as well as fibrous tissue (**Fig. 4a and b**), stained green/blue, whereas the relatively more mature bone in Region III stained red (**Fig. 4d**). This discrepancy could be attributable to the degradation of grafted CaP, which would have increased the local level of minerals in the earlier regions of repair in the implant sights. In the present work, the tissues in Region I and II stained green/blue because of a high concentration of minerals newly released by CaP particles. It could be envisaged that if there had been no implanted CaP, the woven bone of Region II would have stained red.

The red staining of the Region III bone at 40 and 60days (**Fig. 3b and c**) was possibly due to the quick loss of CaP-associated minerals before the new avascular cortical bone was well calcified. Thus, the green/blue staining in the present work did not necessarily indicate a high degree of bone maturation in Region I and II. In fact, the bone tissue in Region II (green/blue) was less mature than that in Region III (red), as indicated by the histological characteristics of sections

stained by H&E (see the next section). However, the red staining of bone in Region III at 40 and 60days (**Fig. 3b** and **c**) indicated that the regenerated bone was still poorly mineralised.

Fig. 5 shows the area percentages of Region I, II and III tissue (excluding the callus areas) as determined by histomorphometric analysis using the samples stained by the Goldner's trichrome method (undecalcified). Compared with the 20-day group, the area percentage of Region III (red) bone in the defects of the 40- and 60-day samples increased to nearly 100 %, with tissues of Region I and II persisting as calli above the normal position of skin.

In short, the present grafted biomaterial was largely decomposed and replaced by new bone after 40 days of implantation, and the defects were nearly filled by newly formed bone. The regenerated dense bone was cortical bone that was markedly less vascularised than at earlier time points.

Analysis of decalcified samples: Hematoxylin-Eosin (H&E)

Fig. 6 demonstrates the histological images of samples retrieved at days 20 and 40 post-implantation. In the 20-daysample (**Fig. 6a**), the tissues of Region I-III presented in an order that was consistent with the growing direction of new bone, i.e., from the border to the centre of the defect. **Fig. 6b** is a consecutive series section of the field shown in **Fig. 3b** (Goldner's trichrome). The histological characteristics of the three regions (I, II, and III) revealed by H&E staining were in agreement with those shown by the Goldner's trichrome method (**Fig. 4**), i.e., the tissue of Region I was non-osseous fibrous tissue mixed with biomaterial particles. The tissue of Region II was newly formed bone populated with many irregular-shaped cavities. The tissue

of Region III was dominated by avascularised cortical bone containing very few irregular-shaped cavities.

Region I stained purple (or red) because of the presence of biomaterial particles. Chitosan appeared completely absorbed and was replaced by the fibrous tissue at day 20 post-implantation. However, the density of biomaterial particles was distributed unevenly in Region I in the 20-day samples (**Fig. 6a**). Large agglomerates of biomaterial particles remained in the central area of the defect (**Fig. 7a**), and few agglomerates were observed in the zone immediately next to Region II (**Fig. 7c**). In between, the amount of biomaterial was reduced in terms of area percentage (**Fig. 7b**). Giant cells were observed surrounding biomaterial particles in Region I (**Fig. 7b and c**).

Many blood vessels were also observed in Region I, especially in the regions represented by **Fig. 7b and c**. Histomorphometric analysis indicated that the area percentages of biomaterial particles in the three typical regions represented by **Figs. 7a-c** were $\sim 40 \pm 5 \%$, $\sim 25 \pm 10 \%$, and $< 2 \pm 0.5 \%$, respectively. The number of blood vessels per unit area in the region represented by **Fig. 7c** was determined to be $\sim 85 \pm 18 / \text{mm}^2$, which is significantly ($p < 0.05$) higher than that in corresponding regions of the control samples at day 20 post-surgery, which was $\sim 55 \pm 14 / \text{mm}^2$.

Fig. 8a demonstrates the transition zone between Region I and II, where new woven bone was growing into the fibrous tissue of Region I and cavities were forming around blood vessels, as marked by the blue arrows. It is apparent that blood vessels within the irregular-shaped cavities in Region II (**Fig. 8b**) were originally formed in the fibrous tissue of Region I. These blood

vessels and nearby undifferentiated (mesenchyme-like) cells were wrapped by new bone, resulting in the irregular-shaped cavities in the primary bone of Region II. The mesenchyme-like cells within these cavities subsequently appeared to have differentiated into osteoblasts, which deposited concentric lamellar bone around the outside of the cavities, resulting in the formation of what we believe to be structures very similar in appearance to primary osteons (Fig. 9a).

A comparison of Fig. 9a with Fig. 9b (Goldner's trichrome stain) reveals that the primary lamellar bone (Zone 1) stained blue, most likely because the structure of lamellar bone was more mature than in woven bone. The woven bone (Zone 2) was likely stained red because of both the immature structure and reduced mineral concentration in lamellar bone in Zone 1. The distal woven bone (Zone 3) stained blue because of the high concentration of minerals.

The formation of putative primary osteons was more advanced in Fig. 8c than in Fig. 8b. In Fig. 8c, as indicated by the concentrically lamellar bone, some putative primary osteons still had blood vessel cavities at its centre (Fig. 9c), whereas no cavities could be observed in others (Fig. 9d). The growth pattern of woven bone, the formation of irregular-shaped primary blood vessel canals, and the generation of primary osteons revealed in Fig. 8 are consistent with the early stage of bone remodelling (30, 31).

In Fig. 8c, biomaterial debris was observed. These irregular-shaped purple areas in between primary osteons appeared to have a high concentration of mineral ions, as indicated by the intense purple staining of these areas in contrast with the red staining of the bone matrix stained.

These purple-stained areas were apparently linked to the formation of primary osteons, which involved re-distribution of bone minerals.

The defects of the 60-day samples were predominately filled with virtually non-vascularised cortical bone (Region III) with a small amount of immature bone (Region II) and very little soft tissue (Region I) (**Fig. 10a**). Although no biomaterial debris could be observed in the 60-day samples, the purple staining due to the unevenly distributed minerals could still be observed around primary blood vessel canals in Region II (**Fig. 10b**), indicating that the remodelling process was still on-going inside the new bone.

3.3. SEM examination

The mineral distribution in Region II was analysed under SEM (**Fig. 11**). Three serial consecutive sections of the same region were examined histologically with Goldner's trichrome (**Fig. 11a**), H&E staining (**Fig. 11b**) and in SEM (**Fig. 11c**), separately. The blue zone around blood vessel cavity (marked as BV₂) in **Fig. 11a** was stained light purple (**Fig. 11b**). The same zone was bright in back-scattered images (**Fig. 11c**). Back-scattered imaging, which is sensitive to the atomic weights, indicated that the bright zone around BV₂ in **Fig. 11c** contains more metal elements, which might be Zn. Elemental mapping suggested that the concentration of calcium (**Fig. 11d**) is somewhat high in the histological (H&E) purple zone, but the sodium concentration is markedly high (**Fig. 11e**), whereas the carbon concentration was lower (darker in **Fig. 11f**). One possible reason could be the formation of hydroxyapatite, Ca₁₀(PO₄)₆(OH)₂, in the primary osteon, which pushes excessive Na⁺ ions out of the osteon, and may form complexes with trace metals.

3.4. TEM examination

Although under optical microscope, few particles were observed in Region I immediately next to Region II in 20- and 40-day samples (**Fig. 7c**), TEM examination revealed that many sub-micro-sized particles presented in putative giant cells in Region I (**Fig. 12a and b**). These sub-micro-sized particles were broken into nano-sized particles (**Fig. 12c**) and had an amorphous structure (**Fig. 12d**).

3.5. Summary of major observations

1. The regeneration process of bone in rat calvaria displayed three major regions. Region I: fibrovascular tissue infiltrated into the biomaterial-grafted region (**Fig. 7**). Region II: new woven bone, i.e., primary bone, regenerated and replaced the tissue of Region I (including the fibrous tissue and degraded biomaterial) (**Fig. 8**). Region III: woven bone restructured into primary lamellar bone (**Fig. 10**).
2. The regenerated cortical bone is primarily avascular.
3. The degradation of CaP particles occurred primarily in Region I. Large agglomerates were first infiltrated and fragmented in the fibrous tissue (**Fig. 7**), and individual particles degraded to become sub-micro-sized ($\leq 1 \mu\text{m}$) particles, which were then phagocytosed by giant cells. Inside the cells, sub-micro-sized particles were further broken down to nano-sized particles by lysosomes (**Fig. 12**).
4. The degradation of CaP stimulated the formation of blood vessels in Region I (**Fig. 7**). In **Fig. 7**, CaP particles degraded slightly in **Fig. 7a** and nearly fully dissolved in **Fig 7c**.

Meanwhile, the density of blood vessels was higher in **Fig. 7c** than in both **Fig. 7a** and **7b**.

5. The bone remodelling process in the biomaterial grafts was similar to that in the femur of rodents (30, 31); i.e., the primary blood vessels that formed in Region I remained in the newly woven bone during its generation, resulting in many large, irregular-shaped cavities in the woven bone of Region II, and the primary blood vessel canals were then encapsulated by primary osteons (**Fig. 8**). However, no secondary osteons and blood vessels (i.e., a Haversian system) were observed.
6. During the remodelling, the re-distribution of minerals occurred, with excessive sodium being pushed out of primary osteons (**Fig. 11**).
7. The 5-mm defect was nearly completely filled with new bone tissue around day 40 post-implantation and healed completely with non-vascularised cortical bone after 60 days post-implantation. The present CaP material was completely degraded and replaced by new bone.

4. Discussion

4.1. Control of healing rate of rat calvarial defects

Bone healing kinetics are controlled by a combination of physical factors (such as availability of physical scaffolds, availability of bone minerals, and degradation rate of implanted biomaterials) and biological factors ones (such as species, age, anatomic position). Histological analysis indicated that Region I of biomaterial repaired rat CSDs was nearly completely replaced by new bone by day 40 (**Fig. 3**). Assuming the bone was growing at a nominally constant rate, the average growing speed of the bone is thus estimated to be $2.5 \text{ mm}/40 \text{ days} = 62.5 \text{ } \mu\text{m}/\text{day} \approx$

$1.0 \times 10^{-3} \mu\text{m/s}$. This rate is remarkably higher than the growth rate of normal remodelling in rats of the same age (Fig. 13). The data in Fig. 13, which was retrieved from literature (32), indicates that the bone growth rate in rats of 4-months old could be as low as several $\mu\text{m/day}$, which has also been reported by another study (33).

An alternative way to quantify growth rate is weight per day. The density of bone is $\sim 2 \text{ g/cm}^3$. The weight of bone in each defect (5 mm-diameter, 0.8 mm-thickness) is $\sim 0.03\text{g}$. The bone growth rate of CaP-grafted defects is thus estimated to be $0.03\text{g} / 40\text{days} \approx 0.8 \text{ mg/day}$. The bone control defects are estimated to have grown at a rate of $0.03\text{g} \times 20\% / 60\text{days} \approx 0.1 \text{ mg/day}$, showing that the implanted defects repair at significantly higher rates than the bone growth rate of normal modelling in rats of the control group.

On the other hand, the present study has also demonstrated that the micro-sized ($\sim 14 \mu\text{m}$) biomaterial particles degraded in fibrous tissue into sub-micron particles, which were then phagocytosed (Fig. 12). The dissolution rate of a CaP particle in fibrous tissue is thus estimated as follows. The dissolution rate of a particle is given by the following:

$$\frac{dM}{dt} = \frac{AD}{h} (C_{\text{surface}} - C_{\text{solvent}}) \quad (1)$$

where M is mass of material dissolved, t is time, D is diffusion coefficient of CaP in soft tissue, (D of inorganic ions in aqueous environment is used as the first approximation, which is of $10^{-5} \text{ cm}^2/\text{s}$ order), A is surface area of the particle, h is the thickness of the liquid film, typically being $1.25 \mu\text{m}$ (34), and C_{surface} and C_{solvent} are the concentration of the material in the liquid film on the surface of the particle and the bulk medium, respectively. On the surface, C_{surface} is the supersaturated solubility of CaP (in the form of Ca^{2+} and/or PO_4^{3-} ions) in body fluid, which is $\sim 5.2 \times 10^{-5} \text{ g/cm}^3$ (35). C_{solvent}

is the concentration of dissolved CaP (in the form of calcium and phosphate ions) in normal body fluid, which is $> 4.8 \times 10^{-5} \text{ g / cm}^3$ (36).

Eq. (1) can be modified as follows:

$$\frac{dV}{dt} = \frac{dM/\rho}{dt} = \frac{AD/\rho}{h} (C_{\text{surface}} - C_{\text{solvent}}) \quad (2)$$

where ρ is the density of CaP. Given that $dV = Adr$, where r is the radius of the particle, Eq. (2) becomes the following:

$$\frac{dr}{dt} = \frac{D}{h\rho} (C_{\text{surface}} - C_{\text{solvent}}) \quad (3)$$

Considering that each layer of particles dissolves on both sides, the dissolving rate of each layer is the double of Eq. (3), that is

$$\frac{dr}{dt} = \frac{2D}{h\rho} (C_{\text{surface}} - C_{\text{solvent}}) \quad (4)$$

A calculation using the above data reveals that the maximal dissolution rate of CaP in ceremonial solution is $dr/dt \approx 2.0 \times 10^{-3} \mu\text{m/s}$. The actual rate could be lower because the concentration of calcium and phosphate ions in the body fluid is likely higher than the minimal level $4.8 \times 10^{-5} \text{ g/cm}^3$, not only because the body fluid (such as cerebrospinal fluid and blood serum) is normally very much supersaturated with $\text{Ca}_3(\text{PO}_4)_2$ (35) but also due to the release of these ions from the grafted biomaterial. Thus, it is reasonable to conclude that the dissolution rate of amorphous CaP particles is approximately the same as the growth rate of healing bone (our measurement). This indicates that the healing rate of new bone in the first 40 days of the experiment was controlled by the dissolution kinetics of CaP particles. It is envisaged that the released Ca^{2+} and PO_4^{3-} ions

from degradation CaP induced the regeneration of the bone matrix, and the associated mechanism directly determined the growth rate of new bone.

However, dissolved CaP did not appear to enhance further bone remodelling because the new bone in the defects remained immature during days 40-60 post implantation, as indicated by the red staining in **Fig. 3**. The slow mineralisation process after 40 days might be attributed to two possible reasons. First, the Ca^{2+} and PO_4^{3-} ions released from CaP could have been significantly reduced in Region III, possibly through the canaliculi (**Fig. 4d**). The second possible reason is associated with the lack of an osteon system in rats, which can slowdown the rate of bone remodelling.

4.2. Comparison of the present observations with the normal remodelling process of calvaria in rats and humans

The histological structures and remodelling process of flat bone in rodents show important differences to those of large animals, such as the human and bovine. In humans, flat bones are sandwich structures, composed of two thin layers of cortical bone and cancellous bone in between. The cancellous bone layer, also called diploë, is the location of bone marrow (28). The cortical bone layer is vascularised via the Haversian system of osteons, where blood vessels are contained within osteocyte canaliculi. The average thickness of (adult) human calvaria is ~ 6 mm, with each compact bone layer and the diploë being ~1.8 mm and ~2.4 mm in thickness, respectively. The bone growth rate in human is typically several μm per day (28).

In rats, the skull bones are not sandwich structured, but rather composed only of cortical bone (37). It has also been reported that the bones of rats and mice lack a Haversian canal system and thus lack Haversian remodelling (7, 31). Although a low level of Haversian canals have been reported for the long bones in rats, it has been consistently reported that thin flat bones (e.g., the skull bone) in rats do not have a Haversian system (37). An investigation into the microcirculation of parietal, scapula and ileum in rats convincingly demonstrated that the skull roofs of rats, which were 0.4-0.8 mm thick, did not contain a bone microcirculation, whereas bone marrow sinusoids and cortical vessels similar to that of long bones were observed in thicker flat bones (e.g., ileum) (37). The microvascular pattern of bones in rats is strongly influenced by the bone thickness, reflecting the mass transportation and metabolic requirements of bone remodelling, with more oxygen diffusion need as density increases. The skull bone tissue in rats is thin enough that it can survive by feeding directly off the periosteal network (rather than by canaliculi), which is shared by the adjacent muscular microvasculature (37).

It is interesting to note that the above threshold thickness of ~0.8 mm for the development of intra-bone vascular networks is reasonably consistent with the values reported from the field of tissue engineering. Under static tissue culture conditions, the maximum thickness of engineered tissue is 0.1-0.2 mm (38). Dynamic cultivations with perfusion of culture medium through the construct, which enhances the convective-diffusive oxygen supply, yield tissues of up to ~1 mm in thickness (38). Hence, it has been predicted that mass diffusion is unable to foster tissue survival without blood circulation if the tissue is thicker than ~1 mm (38). This limitation has been believed to be a critical issue in the engineering of thick, vascularised tissue for clinical applications, especially in the case of high-density tissues like bone (39).

In the present study, the thickness of rat calvaria was in the range of 0.7-0.8 mm. The regeneration of new bone in the CaP-grafted defects occurred in three regions: the formation of fibrovascular tissue, growth of woven bone with irregular-shaped blood vessel cavities, and the temporary formation of primary osteons around the blood vessels. The final product was non-vascularised cortical bone, with no sign of secondary osteon formation. Our histological observations are in agreement with the structure of natural calvaria of rats, and the implanted CaP did not induce further Haversian remodelling in the present model. In addition, the regenerated calvaria bone at 60dayspost-implantation was avascularised cortical type with no cancellous tissue in the defect region. These results are also in agreement with the natural counterpart. It must be mentioned again that once the cortical bone formed around day 40, further mineralisation in the cortical bone was slow and was apparently not being enhanced by the grafted CaP, which is likely to be due a range of factors, including passive diffusion, decrease in the rate of cell proliferation as the wound approaches full regeneration, and delayed replacement of collagen with bone matrix. Longer term follow up of tissue regeneration beyond 60 days may be useful in determining the actual duration of re-mineralization.

Nevertheless, the grafted CaP material greatly accelerated the bone growth rate at the initial stage (up to 40 days), resulting in a rate of 62.5 $\mu\text{m}/\text{day}$ or 0.8 mg/day in the present study, which is remarkably higher than in the normal modelling process in rats of the same age (several $\mu\text{m}/\text{day}$ or 0.1 mg/day). This may have been assisted by the temporary formation of vessels, which would have increased perfusion of the wound, as well as increased diffusion of CaP away from the biomaterial, supporting its biodegradation.

Since their bones heal rapidly, rats and mice have been widely used to investigate bone formation, including angiogenesis/vascularisation in artificial bone materials, such as tissue engineering scaffolds and cements (40-46). We would recommend, however, that the usage of these species, especially the calvarial model, should be limited to the investigation of initial regions of bone formation and/or small flat bones, rather than the more complex process of human osteon. Therefore, we do not recommend that rat or mouse models be suitable for animal studies aimed at the engineering of thick bone for clinical applications, in which vascularisation of the artificial bone matrix is critical. Our result of complete restoration, however, with short term vasculogenesis and complete material degradation, suggests ours to be an ideal tissue engineering strategy for encouraging native bone to repopulate large defects.

5. Summary

This work describes a histological investigation of the healing process of critical-sized defects in rat calvarial bone at both the optical and electronic microscopic levels. The major conclusions are summarised as follows: 1) Implanted CaP biomaterials remarkably accelerated the bone growth rate of the defects at the initial stage, with a growth rate of $\sim 60 \mu\text{m/day}$ or 0.8 mg/day , which is much higher than the bone growth rate (several $\mu\text{m/day}$ or 0.1 mg/day) in non-implanted rats of the same age. 2) The CaP-based graft induced histology similar to primary-osteon remodelling, which were eventually fully replaced by avascular cortical bone, similar to native at calvaria, indicating full defect restoration. 3) The growth rate of new woven bone in the CaP-grafted defects was matched closely to the degradation rate of the biomaterial. Further work

is required to explore the combined osteoinductive and vasculogenic effects of our biomaterial on critical sized defects in other species with vascularised compact bones.

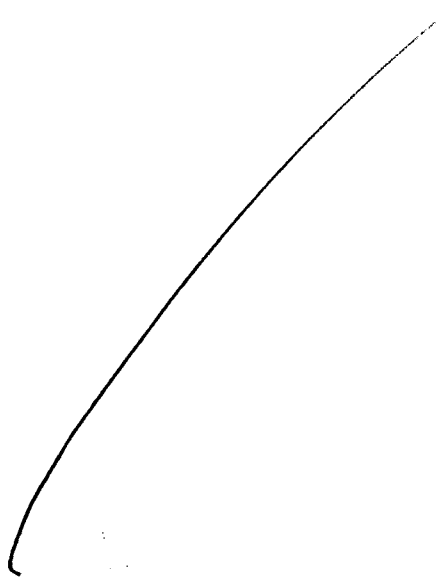
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Figure Legends



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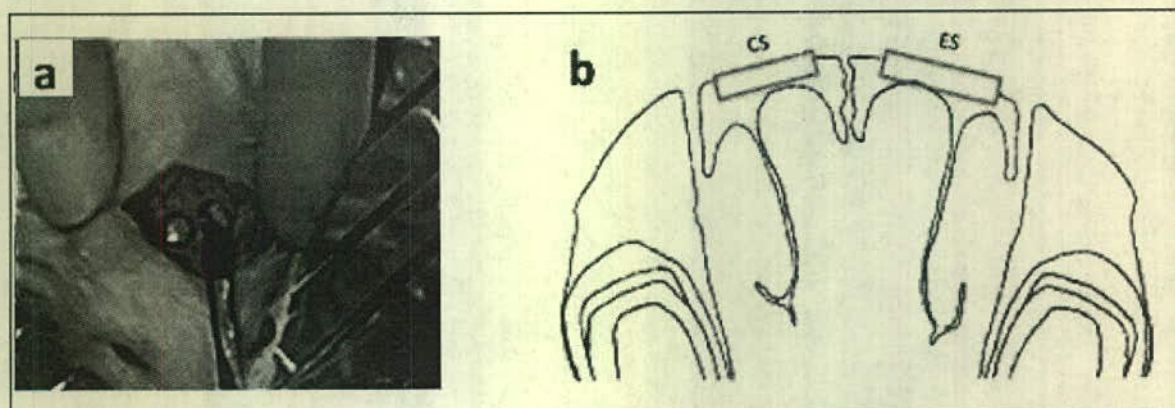


Figure 1 (a) Surgical sites indicated on the exposed rat calvaria and (b) diagram of the coronal view of experimental site (ES-left) and control site (CS-right), which, with surrounding host bone, were retrieved at the end of the experimental period. The histological samples were sectioned across the middle region of each sample along the coronal plane.

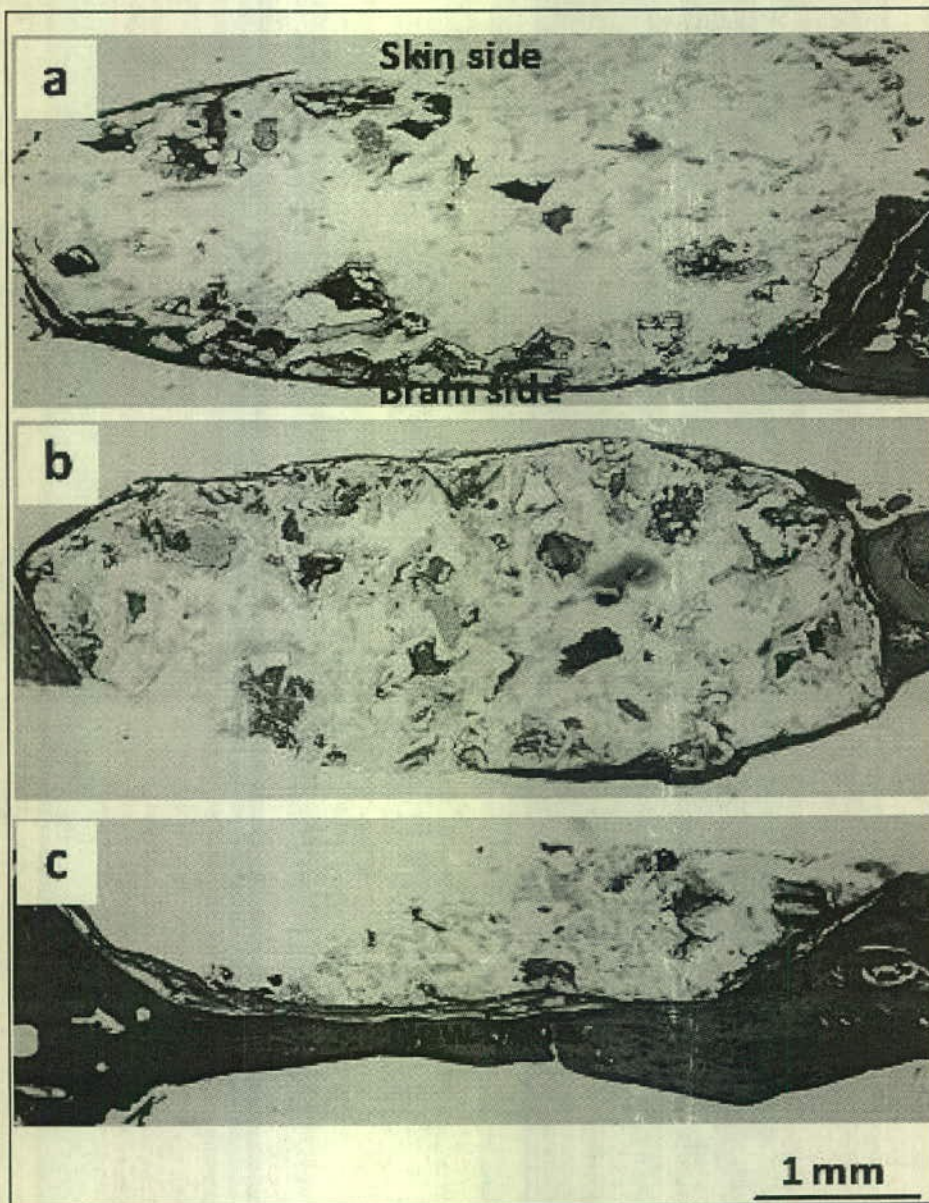


Figure 2 Low-power view of Goldner's trichrome-stained sections of the undecalcified defect tissue from rats that had no implantation of biomaterial particles taken at (a) 20 (b) 40, and (c) 60 days post-surgery. Almost no new bone formation was observed in the defects retrieved at day 20 and 40 post-surgery. After 60 days, new bone was observed, occupying approximately 20 Ar. % of the defect tissue.

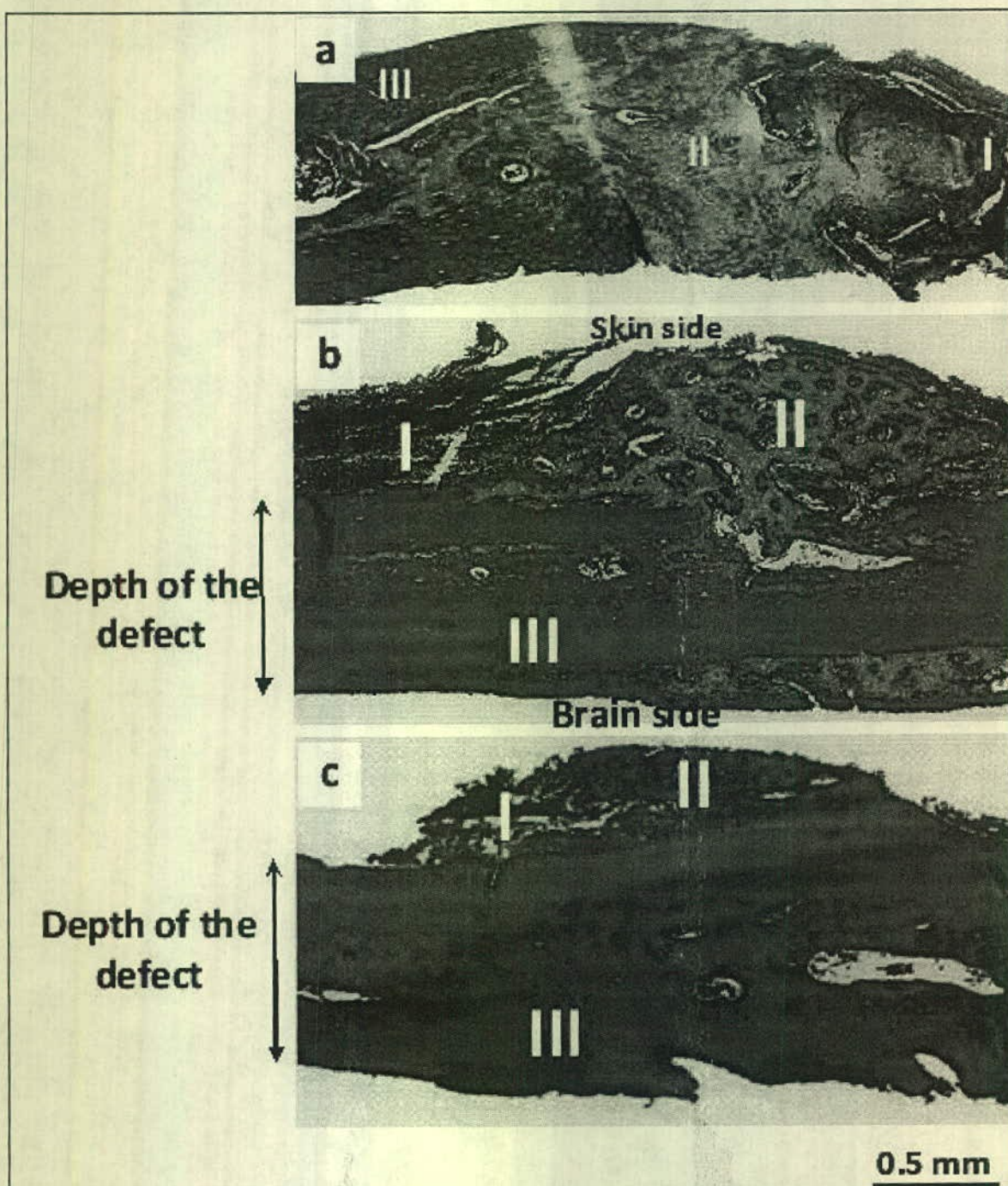


Figure 3 Low-power images of Goldner's trichrome-stained sections of undecalcified CaP biomaterial-implanted tissue at (a) 20, (b) 40 and (c) 60 days after implantation. Tissues in Region I (see Figure 5) consisted mainly of fibrous tissues and CaP particles. Region II consisted of newly formed bone. The blue/green stain observed in Region I and II was due to a high

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concentration of minerals produced by the degradation of CaP particles. Region III consisted of relatively mature bone. The fields of the images covered approximately half of the defects around the centre region.

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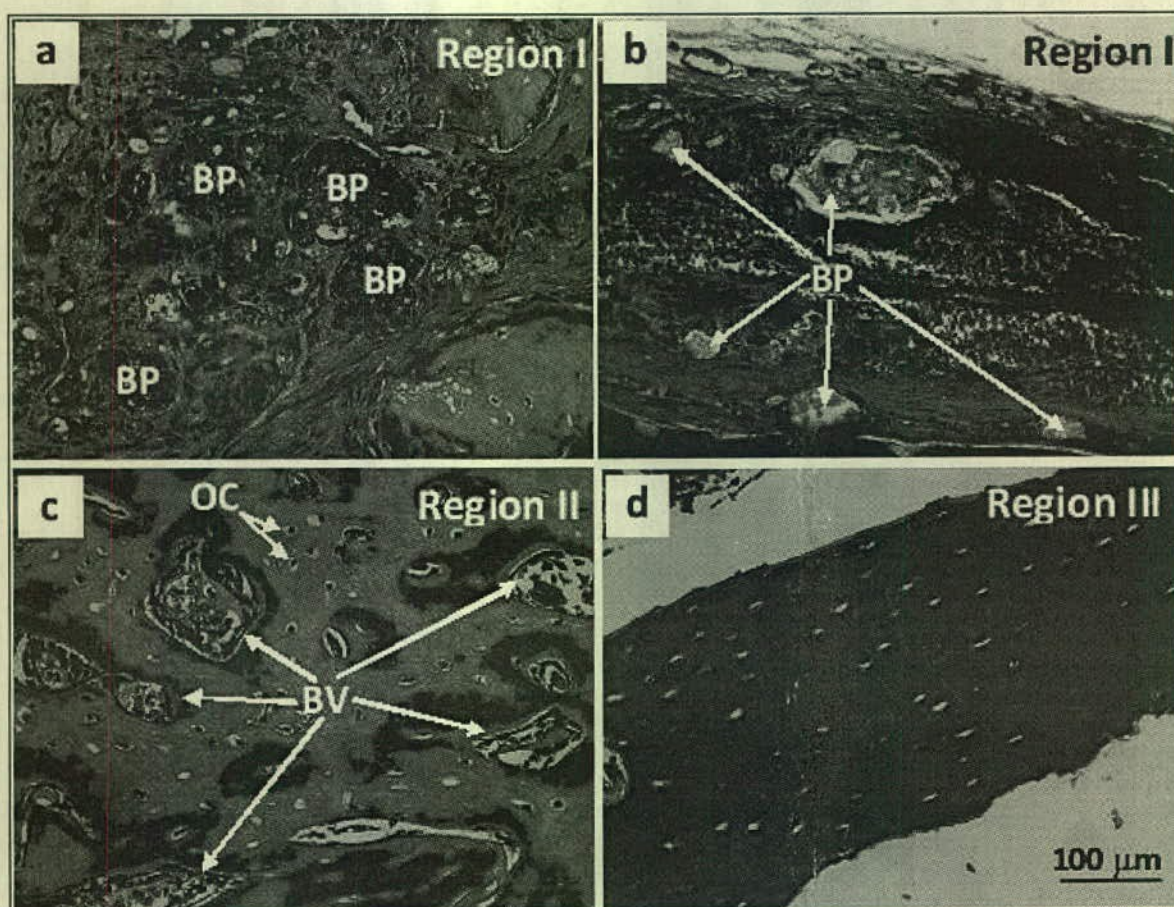


Figure 4 High-power view of Region I, II and III of Fig. 3 (undecalcified). Region I was occupied by the mixture of soft tissue and biomaterial particles (BP), with BP being a particularly predominant tissue component in the 20-day samples (a) and fibrous tissue and blood vessels being the major component in the 40-day samples (b). Region II displayed a great deal of newly formed bone characterised by penetration by many blood vessels (BV); large rounded osteocytes (OC) were also prominent and numerous (c). Region III (b) consisted of cortical bone (red). The green/blue stain in the present samples may have been caused by a high concentration of minerals released by CaP particles, most of which lost quickly before precipitating into crystalline bone hydroxyapatite. The four images have the same magnification.

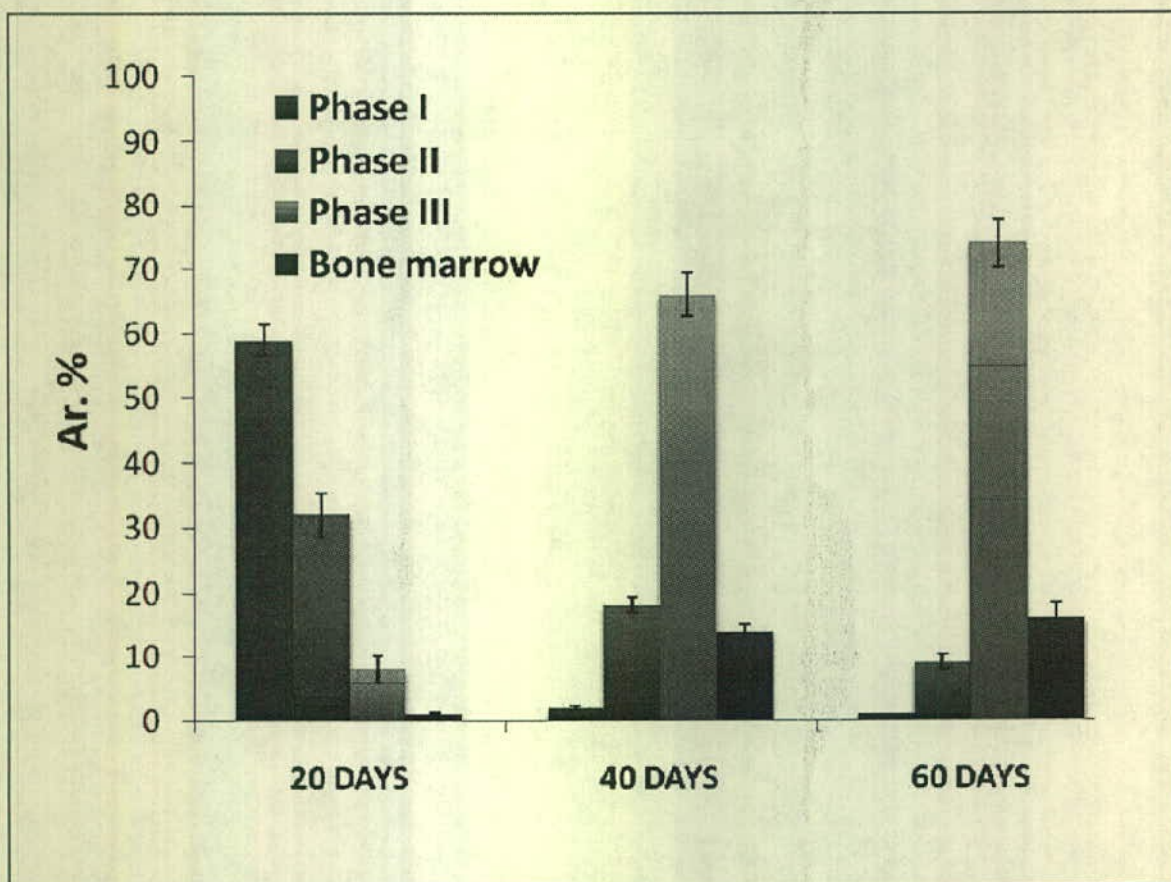


Figure 5 Area percentages of Region I, II and III after retrieval at day 20, 40 and 60.

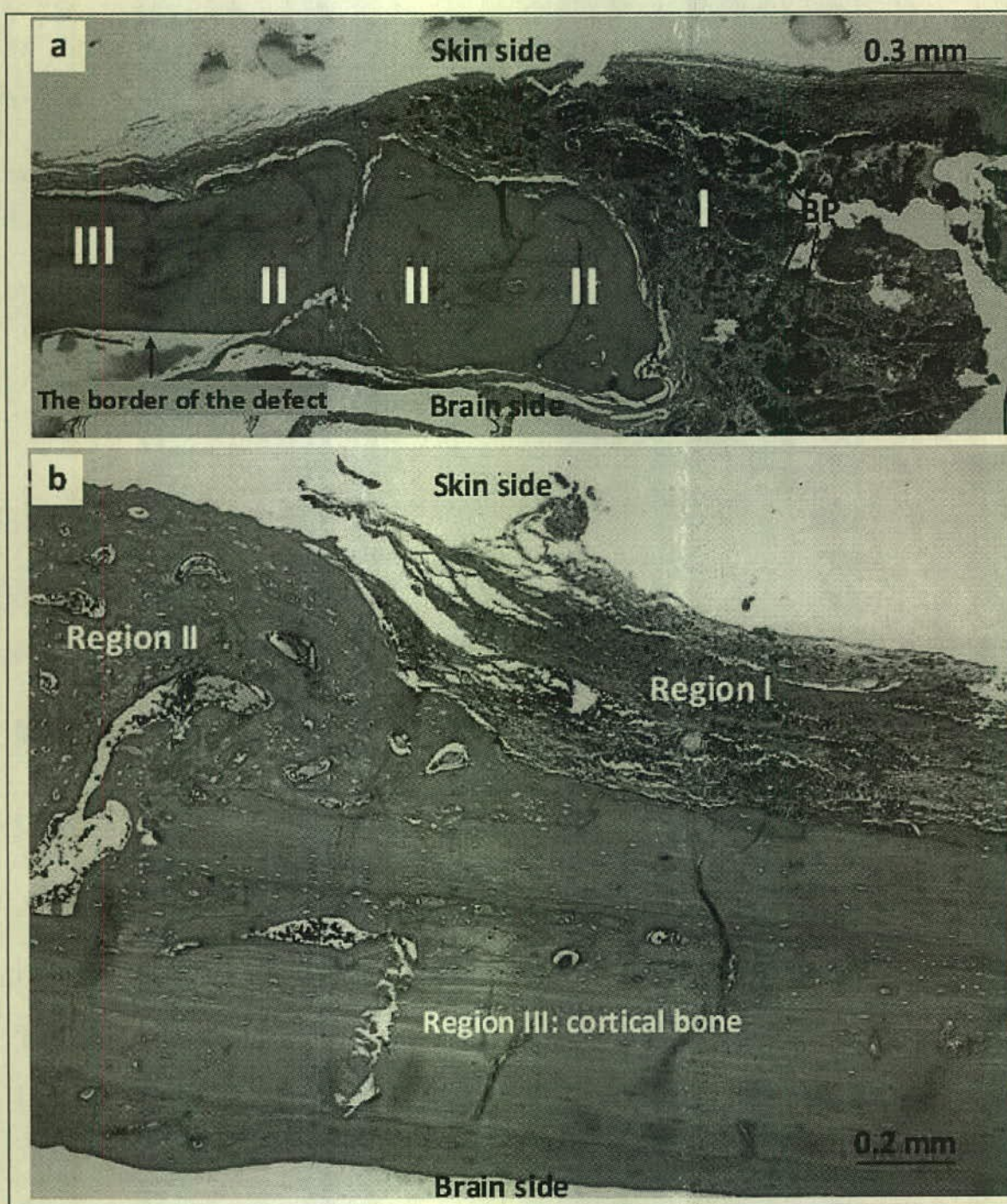


Figure 6 Low-power views of H&E images of CaP biomaterial-implanted defects retrieved at day (a) 20 and (b) 40 post-implantation. Region I was characterised by fibrous tissue mingling with agglomerations of biomaterial particles (BP). Region II was characterised by newly formed

146

bone that still had a relatively high porosity and a large amount of blood vessel penetration. Region III was characterised by mature cortical bone.

59

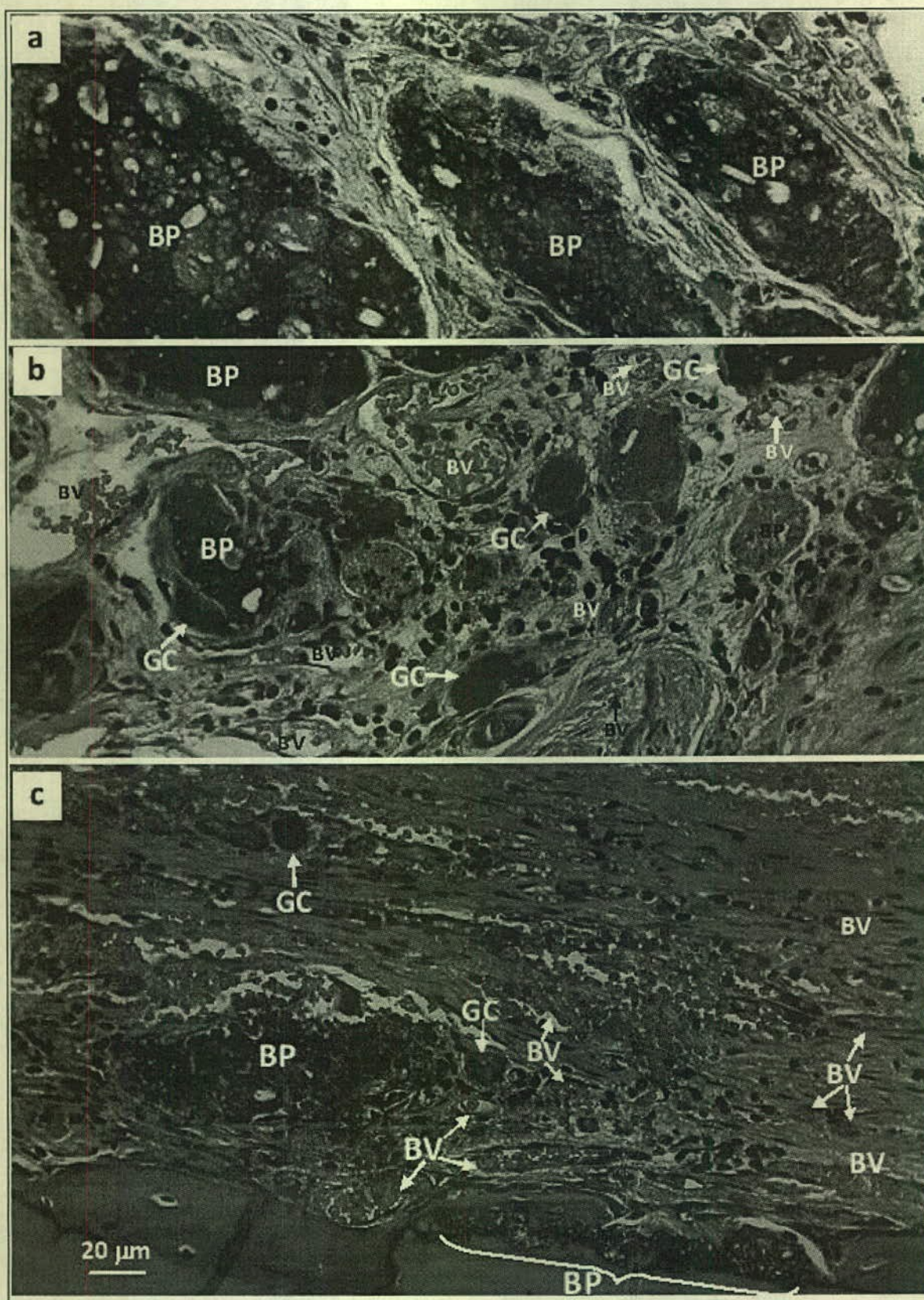


Figure 7 Three representative high-power views of H&E stained tissue in Region I observed in samples of CaP-biomaterial implanted bone defects retrieved at 20 days post-implantation. (a) Agglomerations of bioceramic particles (marked as BP) were surrounded by fibrous tissue. (b) Reduced particles were surrounded by fibrous tissue with prominent blood vessels (BV), where giant cells (GC), presumed macrophage polykaryons consistent with a foreign body reaction were noted in proximity to the BP. (c) The zone was dominated by fibrous tissue.

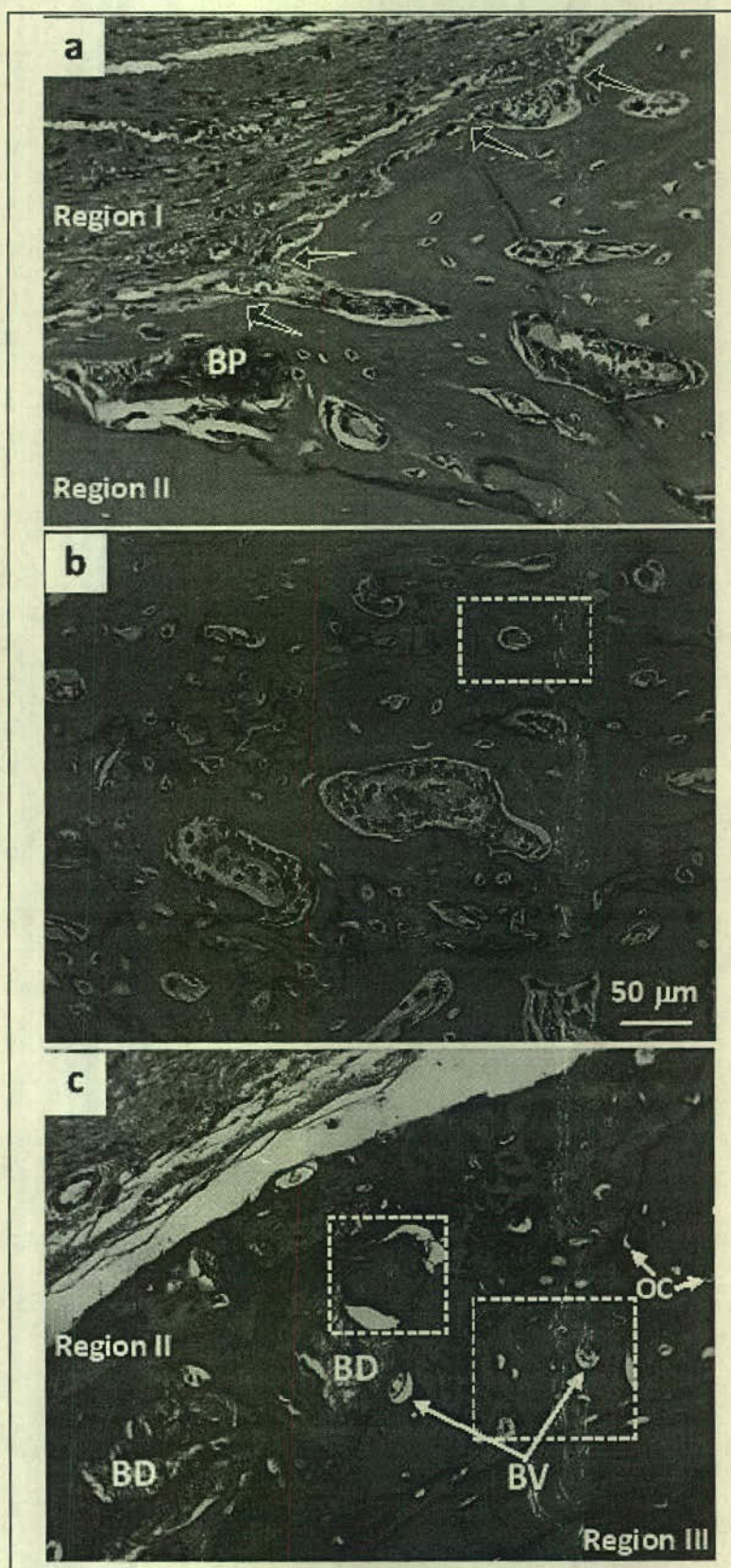


Figure 8 High-power view of H&E-stained decalcified soft tissues of CaP biomaterial-implanted bone defects retrieved at 20 and 40 days post-implantation. (a) The transition zone between Region I and II. The blue arrows indicate the growing directions of woven bone. (b) Many irregular-shaped cavities in Region II. (c) Primary osteons were observed in Region II. OC stands for osteocyte, BV for blood vessel, and BP for biomaterials particle. The three images have the same magnification.

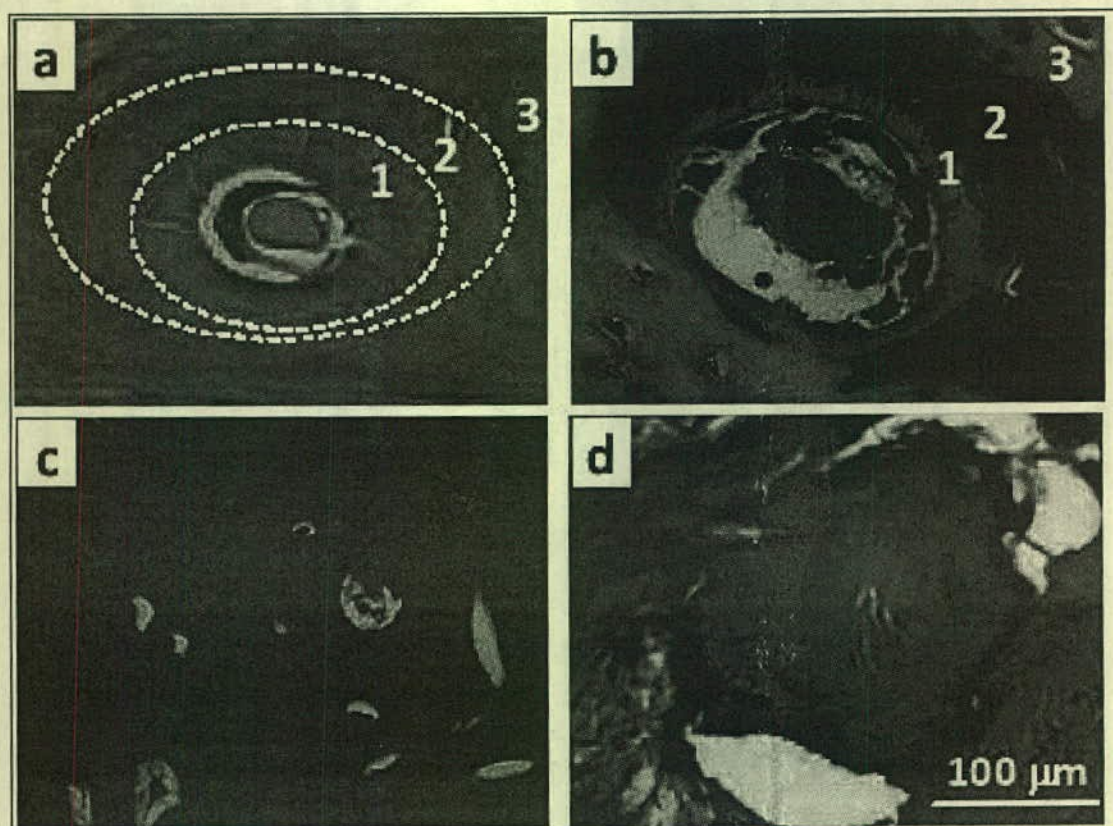


Figure 9 High-power histological images of bones and blood vessel cavities in Region II. (a) The framed area of Fig. 8b, H&E stain, (b) Goldner's trichrome stain, and (c-d) the framed areas of Fig. 8c, H&E stain. The four images have the same magnification. H&E staining applied for decalcified samples and Goldner's trichrome staining for undecalcified samples.

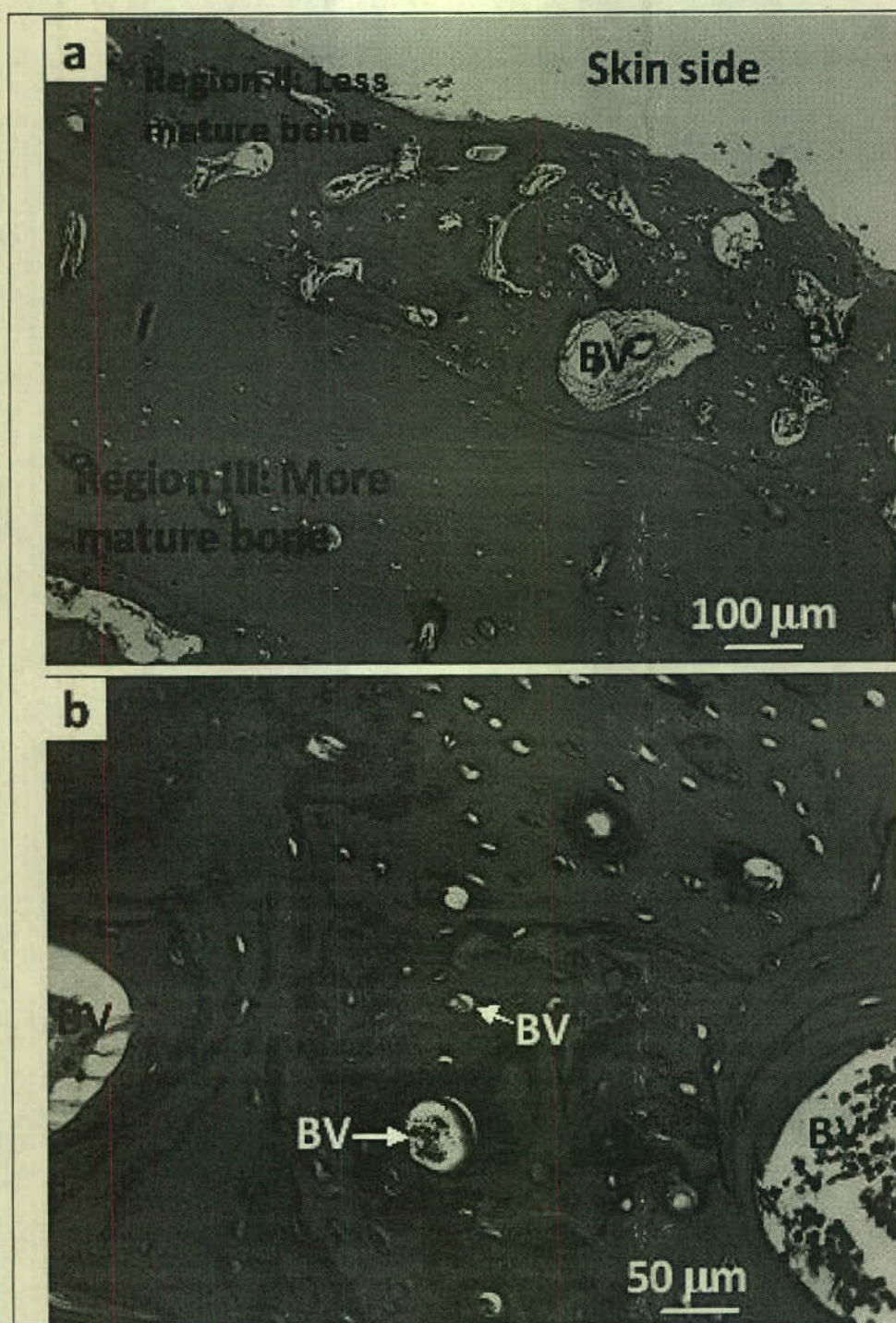


Figure 10 Histological (H&E stained) images of decalcified CaP-implanted defect tissue samples retrieved at day 60 post-implantation. (a) The 5-mm defects were almost completely filled with bone tissue but exhibited two distinct areas. These include areas of newly formed

cortical bone displaying significant porosity with prominent blood vessels (BV), as well as high numbers of large osteocytes. A distinct area delineated by a cement line resembled relatively more mature, low porosity cortical bone. (b) In the area of the less mature bone, biomaterial particles could not be clearly observed, but some irregular areas of purple staining may indicate areas containing remnants of these particles.

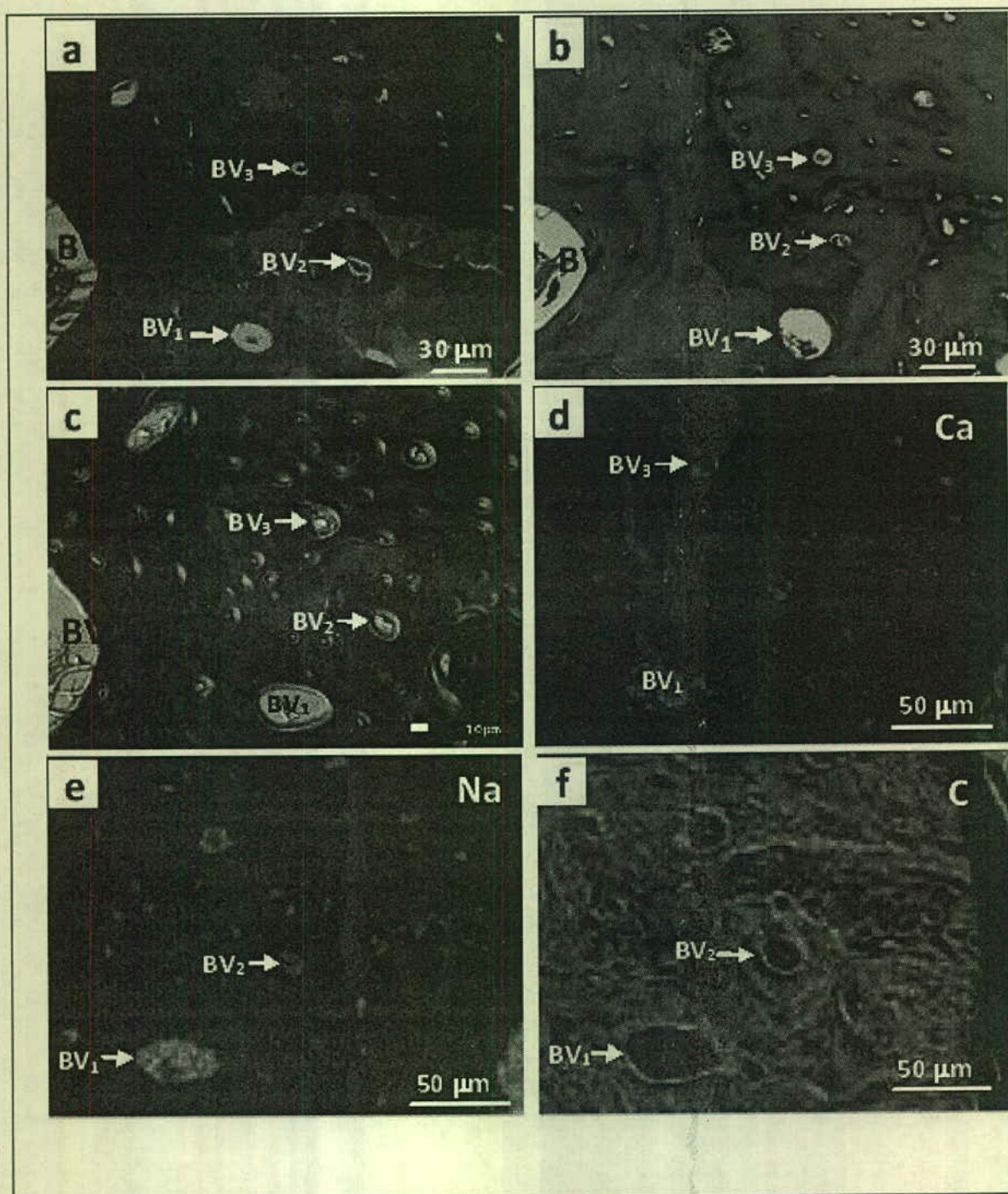


Figure 11 Histological and scanning electron microscope (SEM) images of tissue taken from CaP particle-implanted defects at 60 days post-implantation. (a) Goldner's trichrome, (b) H&E,

(c) electron back-scattered (EBS) image, elemental mapping images of calcium (d), sodium (e) and carbon (f).

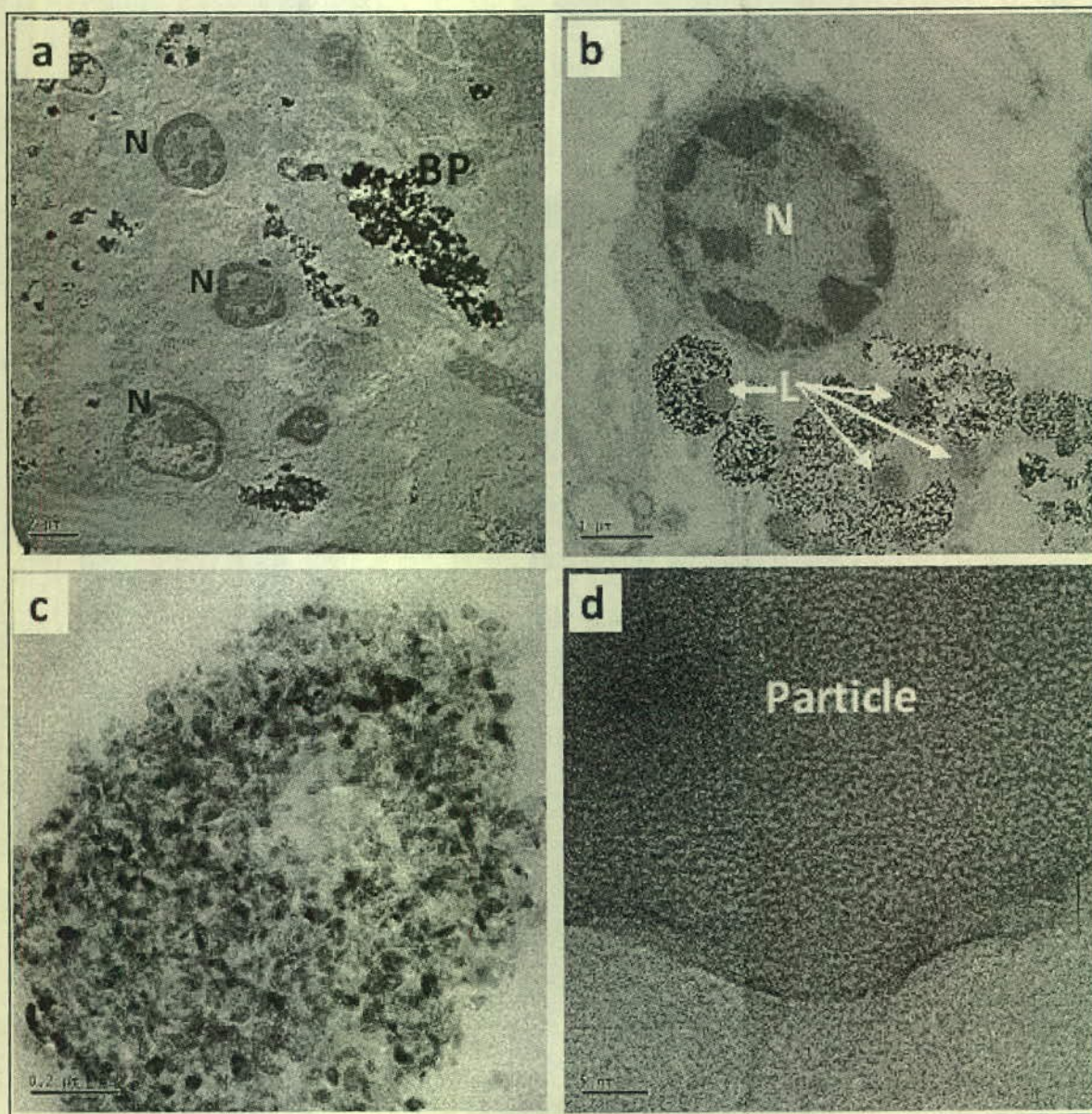


Figure 12 Transmission electron microscope (TEM) images of (a) biomaterial particles inside cells in the transition zone between Region I (fibrous tissue) and II (new bone formation) in samples retrieved at day 20 post-implantation, (b) the particles near the nucleus of the cells, (c) a micro-sized particle had broken down into nano particles, and (d) the high resolution TEM image showing that the nano-sized material was amorphous. L indicates a probable lysosome, and N indicates the cell nucleus. Similar morphologies were also observed in the transition zone between Region I and II in samples retrieved at day 40 (not shown).

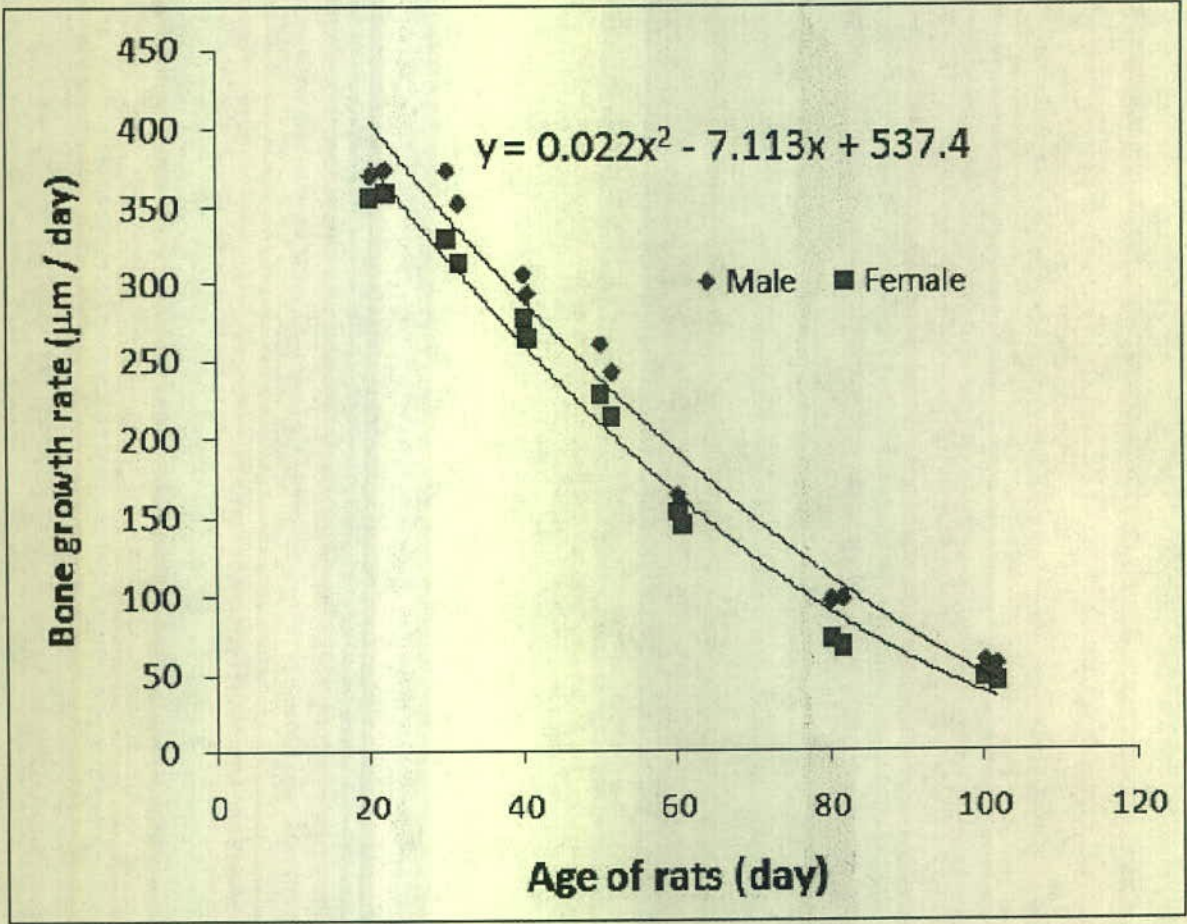


Figure 13 Bone (tibia) growth rate vs age of rats. The equation represents the curve that fits with the data of male rats. Data were retrieved from (33).