Title: PREPARATION METHOD OF POROUS BETA TRICALCIC PHOSPHATE GRANULES

Abstract: Disclosed relates to a preparation method of β-tricalcium phosphate granules and, more particularly, to a preparation method of spherical porous β-tricalcium phosphate granules using tricalcium phosphate precursors, porous precursors, gelatin solution and dispersion medium. The preparation method of the present invention prepares β-TCP granules and uses a dispersion medium to promote the gelation, differently from other methods. The β-TCP granules prepared containing micropores and macropores all together have a biocompatibility. Accordingly, the β-TCP granules grafted in vivo stimulate the new bone formation by acting as a frame, into which new osseous tissues grow to enter, not causing an immunological reaction, thus being effectively used as a bone graft material or a bone scaffold.
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG). For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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Description

PREPARATION METHOD OF POROUS BETA TRICALCIUM PHOSPHATE GRANULES

Technical Field

The present invention relates to a method for preparing porous β-tricalcium phosphate granules.

Background Art

In general, when osseous tissue is damaged due to trauma, tumor, deformity or physiological phenomenon, bone is filled in the damaged region to form new bone. The most general method for the recovery of bone defect includes autogenous bone graft extracting a portion of patient's own bone and transplanting the extracted, allogeneic bone graft transplanting other people's bone treated chemically, and heterologous bone graft transplanting an animal's bone treated chemically. The autogenous bone graft is the preferred method in general, whereas, it has some drawbacks in that it requires a secondary operation, it is difficult to obtain a necessary amount of bone and it is also difficult to carry out the operation in a general medical practitioner's office. Meanwhile, the allogeneic bone graft may cause an immunological reaction and has a risk that may introduce virus such as AIDS, hepatitis, etc., even though there is very little probability. Moreover, the heterologous bone graft may cause the immunological reaction and has some drawbacks caused by various factors, e.g., bovine spongiform encephalopathy (BSE). Accordingly, it is necessary to develop a biodegradable bone graft material that is supplied with sufficient amounts, has no infection possibilities of diseases, has excellent biocompatibility substitutable for existing graft materials and is properly absorbed to be replaced with regenerated bone.

Typical artificial bone materials include calcium phosphate compounds, such as hydroxyapatite (HA), tricalcium phosphate (TCP), etc., bioglass, calcium carbonate and the like.

In such artificial bone materials, hydroxyapatite (HA) and tricalcium phosphate (TCP) are exemplary biomaterials that have attracted attention as artificial bone materials. Hydroxyapatite (HA) is crystallographically and chemically analogous to inorganic components that constitute bones and has is directly bound to bones. However, it has also some drawbacks in that the bone bound at the interface no longer grows to get in due to a low in vivo solubility and is not replaced completely with bone, thus remaining to the end. Tricalcium phosphate has the same properties that it is
directly bound to bones as hydroxyapatite; however, it is dissolved by degrees in vivo and exhausted after all. Tricalcium phosphate is used as artificial bone material, particularly, as bone filler to be filled in the bone defect, like hydroxyapatite. Recently, tricalcium phosphate and hydroxyapatite have complexed to control the in vivo absorption rate. Tricalcium phosphate for the bone filler may be used in the form of close bulk, with a porous structure connected with open pores, or in the form of granule. To use such tricalcium phosphate of close bulk or of porous structure besides the granules as artificial bones, it is necessary to maintain sufficient strengths. Accordingly, tricalcium phosphate is sintered to have a high relative density.

Tricalcium phosphate is harder to sinter than hydroxyapatite and the reasons are as follows. Tricalcium phosphate has β-phase and α-phase polymorphisms, differently from hydroxyapatite that has no polymorphisms, β-phase is a low-temperature phase having a hexagonal crystal structure. If such low-temperature β-phase is heat-treated at a temperature of 1,100 to 1,800 °C, a phase transition occurs to become high-temperature α-phase having a monoclinic system. Since such high-temperature α-phase reacts intensively with water, it is inappropriate to use as a bio material for graft. Moreover, the phase transition from β-phase of high density to α-phase of low density causes minute cracks in the sintered body, thus deteriorating the overall strength of material. For such reasons, tricalcium phosphate of β-phase has been preferred to the others. However, to prepare tricalcium phosphate of β-phase or a sintered complex of hydroxyapatite and tricalcium phosphate of β-phase, the sintering process should be carried out at a temperature below 1,180 °C, the temperature where the phase transition of tricalcium phosphate occurs. Here, since the relative density is shown below 90% at such temperature, it is difficult to prepare a sintered body of high density. Accordingly, to apply tricalcium phosphate of β-phase having excellent bioabsorbability to a biomaterial, it is necessary to prepare a sintered body of high density and practically various sintering methods have been studied to solve such problem.

Reviewing related arts using tricalcium phosphate, Korean Patent No. 26686 has disclosed a method of manufacturing a prosthetic part for use as a body implant comprising the steps of providing a base member, having a porous bone attachment, and a coating material of hydroxyapatite and β-tricalcium phosphate; and applying the coating material directly to the bone attachment, while providing thermal energy thereto. Such method uses hydroxyapatite and β-tricalcium phosphate as the coating material, applies heat more than 1,350 °C to the coating material during the application, and transforms the coating material into bioabsorable α-tricalcium phosphate, thus manufacturing a prosthetic part used as a body implant.

Moreover, Korean Patent No. 95872 has disclosed a medical material and a method
for preparing the same, in which a layer comprising composite particles covered securely with a material having a high biocompatibility is welded on the surface of a material having a high biostability with a high strength through pores interposed.

In addition, Korean Patent Publication No. 2003-6787 has disclosed a chitosan bead containing tricalcium phosphate for bone substitute, wherein the amount of tricalcium phosphate contained is, desirably, 0.1-0.8 parts by weight based on one part by weight of chitosan.

Moreover, U.S. Patent No. 5,017,518 has disclosed a process for producing calcium phosphate ceramics having porous surface. Particularly, the process comprises the steps of: a) preparing untreated calcium phosphate ceramics, which comprises a mixture of hydroxyapatite and tricalcium phosphate, and b) treating the untreated ceramics with an acidic solution to selectively dissolve the tricalcium phosphate in the surface of the ceramics.

Meanwhile, besides the methods for transplanting bone graft materials, there has been proposed a tissue regeneration procedure that separates and cultures cells from a tissue that is to be regenerated and inoculates the cultured cells to an appropriate biomaterial to be amplified and cultured, thus forming a tissue artificially. To inoculate and culture the corresponding tissue cells in such tissue regeneration procedure, a scaffold that is a biomaterial having excellent tissue compatibility and cell adhesiveness is required.

In case of bone regeneration via such tissue regeneration procedure, collagen matrix, poly(glycolic acid) (PGA) mesh, poly(lactic-co-glycolic acid) (PLGA) foam, calcium phosphate ceramics, poly(lactide-co-glycolic acid)/hydroxyapatite (PLGA/HA) and polyphosphazenes have been studied as scaffolds, whereas, there are a variety of problems to be solved.

Reviewing the problems that the scaffolds have, the collagen matrix has a weak strength and is hard to control the absorption rate. Moreover, since it is generally extracted from heterologous or allogeneic individuals, it may cause an immunological reaction. In case of PGA mesh, its sheet is as thin as about 100D. Accordingly, it is difficult to apply it to the case where the bone defect is large and it has weak strength as well. In case of PLGA copolymer, it has a property of hydrophobia, which results in a drawback in that the media is hard to infiltrate and diffuse into the core of scaffold. In addition, the existing ceramics have excellent biocompatibility due to their chemical compositions similar to natural bones; however, their low degradation rate may cause an obstacle to osseous tissue formed newly in the region of graft. Moreover, polyphosphazenes under test stage need further studies for actual applications.
Accordingly, the inventors of the present invention conducting researches aimed at developing a porous bone graft material having a high biocompatibility have prepared porous β-tricalcium phosphate granules that promote the new bone formation via a method of preparing tricalcium phosphate (TCP) using a dispersion medium that promotes the gelation, and completed the present invention.

Disclosure of Invention

Technical Problem

An object of the present invention is to provide a preparation method of porous β-tricalcium phosphate granules.

Moreover, another object of the present invention is to provide a bone graft material or a bone scaffold comprising porous β-tricalcium phosphate granules.

Technical Solution

To accomplish the object of the present invention, there is provided a preparation method of porous β-tricalcium phosphate granules and a bone graft material or a bone scaffold comprising the porous β-tricalcium phosphate granules.

Advantageous Effects

The method of the present invention prepares β-TCP granules and then uses a dispersion medium to promote the gelation, differently from other methods. The β-TCP granules in accordance with the present invention containing micropores and macropores all together have a biocompatibility. Accordingly, the β-TCP granules grafted in vivo stimulate the new bone formation by acting as a frame, into which new osseous tissues grow to enter, not causing an immune reaction. Moreover, the β-TCP granule in accordance with the present invention can be effectively used as a bone graft material or a bone scaffold.

Brief Description of the Drawings

Fig. 1 is a graph depicting X-ray diffraction patterns of pulverized powders of a β-tricalcium phosphate spherical granule of the present invention;

Fig. 2 is an electron microscope photograph of the surface of a β-tricalcium phosphate spherical granule of the present invention taken at 100 x magnification;

Fig. 3 is an electron microscope photograph of a cut surface of a β-tricalcium phosphate spherical granule of the present invention taken at 100 x magnification;

Fig. 4 is a photograph of tissue slice one week after grafting a β-tricalcium phosphate spherical granule of the present invention into the hypodermis of a white rat; and

Fig. 5 is a photograph of tissue slice four weeks after implanting a β-tricalcium
phosphate spherical granule of the present invention into the cranial median section of a white rat.

[29] Best Mode for Carrying Out the Invention

[30] The present invention provides a preparation method of porous β-tricalcium phosphate granules and, more particularly, the method comprises the steps of:

[31] (a) arranging a gelatin solution by admixing water and gelatin powders;

[32] (b) arranging a tricalcium phosphate (hereinafter, referred to as 'TCP') slurry by admixing TCP precursor powders and porous precursors and adding the gelatin solution thereto;

[33] (c) forming spherical granules by adding the mixed TCP slurry to a dispersion medium being stirred and gelating the granules;

[34] (d) separating and washing the gelated spherical granule surfaces with an organic solvent; and

[35] (e) calcining and sintering the washed spherical granules to remove additives other than the TCP.

[36] Moreover, the present invention provides a bone graft material or a bone scaffold comprising porous β-tricalcium phosphate granules.

[37] Hereinafter, the above method comprising such steps of the present invention will now be described step by step.

[38] In step (a), the gelatin solution is arranged by admixing water, desirably, distilled water with gelatin powders.

[39] The gelatin powders may be used by purchasing those commercially available and made of skins of pig or cattle. Since the gelatin solution exists in the form of sol at high temperature and is gelated if the temperature falls, it is desirable to prepare the gelatin solution by adding 6-20 parts by weight of gelatin powders to 100 parts by weight of water, preferably, 6-10 parts by weight, and most preferably, 8 parts by weight and applying heat of 30 to 80°C to the resulting mixture being stirred.

[40] In step (b), the TCP slurry is prepared by admixing TCP precursor powders and porous precursors and adding the gelatin solution thereto;

[41] The TCP precursor powders may be used by purchasing those commercially available or by preparing via an ordinary preparing method. As porous precursors, macromolecules having a size of about 90-100 D may be used or any materials may be adopted if those can be removed via a heat treatment in a calcining process, which will
be described below, evaporated or degraded to gases at 200 to 600 °C, thus forming pores in the space occupied by porous precursors. Desirably, it is possible to use organics, such as hollow spherical macromolecules, naphthalene, starch, etc., and most preferably, hollow spherical macromolecule powders of vinylidene chloride/acrylonitrile (PVDC) copolymer having a density below 0.1 g/D, an average particle size of 60-90 D and an external wall thickness of about 0.5-1.5 D.

It is desirable to add 1-5 parts by weight of porous precursors to 100 g of TCP precursor powders. After mixing uniformly, 12-17 parts by weight of the mixture of TCP precursor powders and porous precursors are added to 100 D of the gelatin solution and stirred to prepare a TCP slurry. Here, the stirring rate is desirably 200 to 600 rpm, and preferably, 400 rpm.

In step (c), the mixed TCP slurry is added to a dispersion medium being stirred to form spherical granules and the spherical granules are gelated.

As the dispersion medium, it is desirable to use a solvent that does not react with water, preferably, mineral oil or corn oil, and most preferably, corn oil that is commercially available. Moreover, since the volume of spherical granules may be varied according to the viscosity of slurry and the stirring rate of dispersion medium, it is possible to regulate the diameter of spherical granules by controlling the two conditions. In the present invention, the above conditions are controlled to prepare the granules having a diameter of 1.4-2.0 D, 1.0-0.6 D, 0.6-0.4 D, or 0.4-0.25 D, the diameter being appropriate to be used as a graft material.

To granulate the TCP slurry, a dispersion medium of room temperature is used firstly and a dispersion medium kept in cold storage is secondly used. The dispersion medium of room temperature being stirred at 100 to 1,000 rpm is admixed with the TCP slurry and continuously stirred to disperse the TCP slurry in the spherical shapes in the dispersion medium. To rapidly gelate the spherically dispersed TCP slurry, a dispersion medium having a temperature (0-20 °C) lower than the room temperature is added thereto, thus obtaining gelated spherical granules ultimately. Here, it is desirable to add the first dispersion medium of room temperature 1 to 3 times more than the volume of the TCP slurry and add the second dispersion medium kept in cold storage 4 to 5 times more than that of the TCP slurry.

In step (d), the gelated spherical granule surfaces are washed using an organic solvent to remove moisture and dispersion medium thereon.

To separate the spherical granules from the dispersion medium, it is desirable to use an organic solvent that reacts with oils to be mixed, preferably, chloroform, ether, hexane, ethanol, acetone or their mixed solvent, and most preferably, hexane and
acetone. Using a water-jet pump, the dispersion medium remaining on the granule surfaces can be washed rapidly. If using the organic solvent like the above, it is possible to remove the dispersion medium, promote the gelation of spherical granules and evaporate the remaining moisture.

In step (e), the washed spherical granules are calcined and sintered to remove moisture, organic solvent and residual organics and cause a phase transition of TCP precursors to β-TCP.

The spherical granules are heat-treated at 1,000 to 1,180 °C under a heating rate of 0.15 to 1.10 °C/min to cause the phase transition of the TCP precursors to β-TCP. Prior to the heat treatment at 1,000 to 1,180 °C, it is possible to comprise the following steps additionally:

- removing moisture at 30 to 50 °C;
- removing moisture and organic solvent at 250 to 350 °C; and
- removing residual organic components at 570 to 670 °C.

First, the spherical granules are dried at 30 to 50 °C or at room temperature using a dry oven to remove the remain moisture and this step is carried out for 3 to 5 hours, desirably. Next, the dried spherical granules are calcined by raising the temperature up to 250 to 350 °C and 570 to 670 °C under the heating rate of 0.15 to 1.10 °C/min to gelatin, porous precursors and organic solvent. Preferably, after calcining the granules by raising the temperature up to 300 °C under the heating rate of 0.25 °C/min for 2 to 4 hours, the temperature is raised up to 620 °C under the heating rate of 0.25 °C/min and maintained for 4 to 6 hours. Subsequently, in order to cause a phase transition of PCT precursors to β-TCP, the temperature is raised up to 1,000 to 1,180 °C to be calcined. Desirably, the temperature is raised up to 1,000 to 1,180 °C under the heating rate of 1.0 °C/min, and preferably, the temperature is raised up to 1,080 °C. The ultimate temperature and time for such calcination are set at those where micropores are present in the β-TCP granules, below the temperature and time where a phase transition from β-TCP to α-TCP occurs. It is desirable to carry out the calcination for 11 to 13 hours, preferably, for 12 hours.

As a result of observation and analysis of the β-TCP granules in accordance with the present invention, micropores and macropores are all present and there is contained no carbon, hydrogen, nitrogen, sulfur or organics in the spherical granule. Moreover, as a result of measuring the safety of the β-TCP granules with fibroblastoma extracted from connective tissue of a mouse, the cell proliferation inhibitory rate is shown as -10%, which may not cause any problems in terms of safety. If grafting the β-TCP granules in laboratory animals, there are no inflammations seen with naked eyes or
using a microscope. Accordingly, it is possible to use the \( \beta \)-TCP granules in accordance with the present invention without any problems in terms of immunology. In addition, if forming a bone defect between the cranial sutures in a laboratory animal and grafting the \( \beta \)-TCP granule in accordance with the present invention, collagen fibers are filled in the area of the bone defect; capillaries newly proliferate in the vicinity of the grafted material and in the connective tissue; osteoblast cells are divided, osteoid tissue and immature bone are formed in the grafted material having porosity; and the new bone formation is increased in the area contacting with the grafted material. Furthermore, the new bone, the grafted material and the connective tissues are present all together with the regions where the connective tissues survive is shown between the new bone and the grafted material. In addition, since the \( \beta \)-TCP granule does not show a genetic toxicity, the \( \beta \)-TCP granule in accordance with the present invention can be effectively used as a bone graft material or a bone scaffold for stimulating the bone regeneration.

The bone graft material or the bone scaffold of the present invention may be prepared by additionally containing at least one component showing an identical or similar function to the \( \beta \)-TCP granule in accordance with the present invention.

Mode for the Invention

Hereinafter, the present invention will now be described more fully with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

Example 1: Preparation of \( \beta \)-TCP Granules of the Present Invention

To prepare \( \beta \)-TCP granules of the present invention, TCP precursors were synthesized primarily as follows.

770 Dof 0.4 M calcium nitrate \( \text{Ca(NO}_2\text{)}_2\cdot4\text{H}_2\text{O} \) solution was prepared as a calcium source. While stirring the prepared solution, 2,000 Dof 0.154 M ammonium phosphate \( \text{NH}_4\text{HPO}_4 \) as a phosphate source was dropped at a fixed speed using a peristaltic pump to be precipitated. Here, to keep the pH at 7.4, a sodium chloride (NaOH) solution and a hydrochloric acid (HCl) solution were used as basic and acidic solutions, respectively. After the reaction, the mixed solution was ripened at a warm room of 37 °C for 24 hours. Precipitates separated from the ripened solution using a
water-jet pump were washed and filtered with triple-distilled water, thus obtaining end precipitates. The end precipitates were completely dried, pulverized and sieved, thus preparing TPC precursor powders.

[70]

1-2: Preparation of TCP precursor spherical granules

TCP precursor spherical granules were prepared using the TCP precursor powders obtained in Example 1-1.

[71]

TCP precursor powders prepared in Example 1-1 were admixed uniformly with 30 Dof hollow spherical macromolecule powders of vinylidene chloride/acrylonitrile (PVDC) copolymer (Expancel microspheres manufactured by Expancel Inc. Sweden) as porous precursors having an average particle size of about 80 Dand an external wall thickness of about 1 d. In addition, 8% gelatin solution was prepared to be kept at about 60°C. 700 Dof gelatin solution was added to the resulting powders mixed uniformly with TCP precursor powders and porous precursors and mixed to prepare a TCP precursor slurry.

[72]

The TCP precursor slurry was added to about 1500 Dof corn oil of room temperature being stirred at 400 rpm to prepare spherical granules. Next, to promote the gelation of granules, about 3,000 Dof corn oil kept in cold storage was added thereto under the same stirring condition. After separating the TCP precursor spherical granules gelated in the corn oil using a filter, the TCP precursor spherical granules were washed with hexane to remove the corn oil remaining on the surfaces of granules using a water-jet pump. Further, acetone was used to wash the TCP precursor spherical granules again for the purpose of promoting the gelation and removing the remaining moisture, thus obtaining TCP precursor spherical granules. The still remaining moisture was dried at room temperature.

[73]

1-3: Calcining and Sintering

The TCP precursor spherical granules obtained in Example 1-2 were calcined in an electric furnace to prepare end TCP spherical granules. Since additives used during the preparation of TCP precursor spherical granules may be left according to heating rates, the calcination process was carried out in several stages.

[74]

Moisture and organic solvent were removed at 300°C, and gelatin and spherical precursors were removed at 620°C. Moreover, in order to completely remove the gelatin of large molecular weight, the heating rate was set at 0.25°C/min. Subsequently, the residual organic components were completely removed at 620°C for 5 hours. To completely synthesize TCP spherical granules of compact frame from the TCP precursors and to prevent the micropores from vanishing completely, the resulting TCP precursor spherical granules were heat-treated at 1,080°C, raised at a heating rate
of 1 °C/min, for 12 hours, and cooled slowly, thus obtaining end TCP spherical granules.

Synthesized TCP was confirmed via an analysis of X-ray diffraction as shown in Fig. 1 and the surface and cut surface of the TCP spherical granules obtained in the above manner were observed through a scanning electron microscope (SEM) and depicted in Figs. 2 and 3. In addition, the contents of organic components in the spherical particles were measured using an elemental analyzer and listed in Table 1 below.

Table 1
Content analysis of organic components via elemental analyzer

<table>
<thead>
<tr>
<th>sample</th>
<th>C(%)</th>
<th>H(%)</th>
<th>N(%)</th>
<th>S(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D.: Not Detected

As depicted in Fig. 1, X-ray diffraction patterns having no secondary phases were shown, from which it could be confirmed that complete TCP was synthesized.

Moreover, as depicted in Fig. 2, it could be observed via an electron microscope that spherical granules were prepared and, as depicted in Fig. 3, it could be confirmed from the cut surface of the spherical granule that the micropores and macropores existed all.

In addition, as shown in Table 1, it could be learned that there was contained no carbon, hydrogen, nitrogen, sulfur or organics in the spherical granule.

Examples 2 to 9: Preparation of β-tricalcium phosphate (TCP) spherical granules
Preparation conditions of β-TCP precursor spherical granules in Example 1-2 were varied to prepare β-TCP spherical granules in Examples 2 to 9.

Except for the concentration and amount of gelatin solution and the stirring rate, β-TCP spherical granules were prepared in the same manner as Example 1. The conditions according to the respective Examples were listed in Table 2.

Table 2

<table>
<thead>
<tr>
<th>condition</th>
<th>concentration</th>
<th>amount(ill)</th>
<th>stirring rate(rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example 1</td>
<td>8</td>
<td>700</td>
<td>400</td>
</tr>
<tr>
<td>Example 2</td>
<td>6</td>
<td>700</td>
<td>400</td>
</tr>
<tr>
<td>Example 3</td>
<td>10</td>
<td>700</td>
<td>400</td>
</tr>
<tr>
<td>Example 4</td>
<td>8</td>
<td>500</td>
<td>400</td>
</tr>
</tbody>
</table>
As shown in Table 2, only the concentrations of gelatin solution of Examples 2 and 3 varied from that of Example 1, the amount of gelatin solution of Examples 4 to 7 varied from that of Example 1, and the stirring rate of Examples 8 and 9 varied from that of Example 1.

As a result of preparing β-TCP spherical granules under the respective conditions, there were not shown wide differences between the diameters of the spherical granule particles, except for those of Examples 4 and 8. In Example 4, where the amount of gelatin solution was smaller than the others, the viscosity of TCP slurry was increased, which resulted in the increase of the particle volumes. In Example 8, where the stirring rate was lower, the particle volumes were also increased.

Accordingly, it could be learned that it was possible to regulate the volumes of granule particles by controlling the viscosity of TCP slurry and the stirring rate.

Experimental Example 1: Safety Examination of β-TCP Spherical Granules of the Present Invention

The following experiment was carried out to examine the safety of β-TCP spherical granules prepared in Example 1 above via a cell proliferation test.

1-1: Cell culture

Cells used in this test were fibroblast L929 cells (ATCC No. CCL-I) derived from mouse connective tissue, obtained from American Type Culture Collection (ATCC). Culture was carried out in an incubator under the conditions (temperature: 37 °C, humidity: 95% and CO2: 5%) using a minimal essential medium (MEM) with 10% fetal bovine serum (FBS) via an ordinary method. The fibroblast cells were cultured in 75 Dcell culture flask. Culture solution was changed twice a day. If the cells were cultured good enough to form a monolayer, the cultured cells were subcultured 1:10.

1-2: Test of cell proliferation inhibition

Each 20 Dof physiological saline for 4 g of β-TCP spherical granules was added to
the granules and shaken in a water bath of 37 °C for 72 hours, thus preparing a test solution of β-TCP spherical granule. The cells, monolayer-cultured using the cell culture flask in Experiment Example 1-1, were treated with trypsin-EDTA to collect cells. Then, the cells were counted using a hemocytometer to prepare a cell suspension containing 106 cells for 1 Dof culture solution. Moreover, the test solution of β-TCP spherical granule was mixed commensurately with the culture solution to prepare a β-TCP spherical granule processed culture solution, and the culture solution was mixed commensurately with distilled water to prepare a control culture solution.

Each 0.2 D(2x10^5 cells) of the cell suspension prepared as above was put into fifteen test tubes, wherein each 2 Dof β-TCP spherical granule processed culture solution was added to five test tubes (an experimental group) and each 2 Dof control culture solution was added to the other ten test tubes (five assigned to a negative control group and the other five assigned to a positive control group 2). Here, the five test tubes assigned to the negative control group were centrifuged to remove the culture solutions. Then, the cells were resuspended with phosphate buffer physiological saline of pH 7.0. The resulting suspension was centrifuged twice again to wash the cells and kept at 4 °C, thus preparing the negative control group.

The cells in the ten test tubes of the experimental group and the positive control group were moved to a 24-well plate and cultured in CO2 incubator for 72 hours.

After destroying the cells in the experimental group and the positive control group, the total protein content was determined by the Lowry method of discoloration and the average absorbances of the respective groups were measured, thus calculating the cell proliferation inhibition rate by the following Formula 1.

The results were depicted in Table 3 below.

[Formula 1]

Cell proliferation inhibition rate (%) = \[1 - \frac{\text{average absorbance of experimental group} - \text{average absorbance of negative control group}}{\text{average absorbance of positive control group} - \text{average absorbance of negative control group}}\] x 100

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Test group</th>
<th>rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Experimental group</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>group</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>group</td>
<td></td>
</tr>
<tr>
<td>Absorbance</td>
<td>0.729</td>
<td>0.709</td>
</tr>
<tr>
<td></td>
<td>0.514</td>
<td>-10</td>
</tr>
</tbody>
</table>

[HO]
As shown in Table 3, it could be confirmed that the β-TCP spherical granules in accordance with the present invention had no effects of cell proliferation inhibition on the L929 cells.

Experimental Example 2: Graft Experiment of β-TCP Spherical Granules of the Present Invention

The following experiment was carried out to examine the surrounding tissues and the local reactions occurring when grafting the β-TCP spherical granules of the present invention in vivo.

2-1: Breeding of laboratory animals

Healthy male white Sprague-Dawley (SD) rats weighed about 250 g were used in this experiment. They were bred with ordinary hard foods and drinking water was freely supplied to them.

2-2: Graft Test

Bone fragments used in this test was arranged with dimension (heightxdiameter) of 1 DxD10 Dand treated by radiation sterilization. Moreover, the β-TCP spherical granules prepared in Example 1 were treated in the same manner as Experimental Example 1-2.

After anesthetizing the white rats deeply with a mixture of ketamine (Ketara 10 D/D) and 2% xylazine (Rompun 0.15 D/D), hairs on the skin of the back were removed and disinfected with disinfectant. To each rat, about 5 Dof the skin was incised and then hypodermis was undermined to secure the space for grafting the bone fragment. The bone fragment was grafted spaced at least 1 Dapart from the incised part and sutured. After sacrificing the rats three-by-three by twisting neck after one week, two weeks and eight weeks from the grafts, the surrounding tissues including the grafted bone fragments were collected immediately and fixed in 10% neutral buffered formalin. After fixing the collected ones in the same fixative for two days, the collected ones were decalcified with 5% nitric acid for three days. Subsequently, the grafted bone fragments were dehydrated with ethylalcohol according to an ordinary method, substituted for xylene and embedded in paraffin.

The embedded tissues were cut 4 D thickness using a microtome and then subjected to hematoxylin-eosin staining. After a lapse of one week, the test results were observed with naked eyes and using an optical microscope.

The results were depicted in Fig. 4.

As a result of observing the corresponding tissues with naked eyes, there were found no particular inflammations and the grafted bone fragments were surrounded by thin and transparent tissues.

Moreover, as a result of observing the corresponding tissues using an optical
microscope, the surface of the grafted bone fragment was surrounded by surrounding
tissues of granulation tissue type, the fibrous tissues were weak, and the blood vessels
and numerous cells were filled around and in the grafted bone fragment. Furthermore,
in the vicinity of the grafted bone fragment, there were founded some multinucleated
giant cells. Most of the grafted bone fragments had decalcified surfaces. It was found
during the tissue preparation that the sizes of the grafted bone fragments were
decreased due to the decalcification by nitric acid.

Experimental Example 3: Efficacy Experiment of β-TCP Spherical Granules of the
Present Invention as a Bone Graft Material

The following efficacy experiment of the β-TCP spherical granules of the present
invention was carried out to analyze the biological reactions of osseous tissues for TCP
and to examine clinical efficacy as a bone graft material.

3-1: Breeding of laboratory animals
Healthy male white Sprague-Dawley (SD) rats weighed about 250g were used in
this experiment. They were bred with ordinary hard foods and drinking water was
freely supplied to them.

3-2: Grafting in animals
After anesthetizing the white rats deeply with a mixture of ketamine (Ketara 10 D/D)
and 2% xylazine (Rompun 0.15 D/D), depilation and preoperative aseptic process
(rubbed with 10% povidone-iodine and wiped with 70% alcohol) were carried out
according to an ordinary method. Subsequently, to each rat, the cranial median section
was incised to lift full thickness flap and then the periosteum was cut carefully to be
separated from the surface of cranium. A defect section was formed between two
cranial sutures by a hand trephine bur, injecting a saline solution thereto to prevent
heat generation. Here, in a negative control group, nothing was filled therein and
sutured as it was, whereas, in an experimental group, TCP (0.4-0.6 D) treated by
radiation sterilization was grafted and the periosteum and skin were sutured re-
spectively.

3-3: Tissue Preparation
After sacrificing the rats of the respective groups after one week and four weeks
from the grafts, temporal bones were extracted to be fixed in Bouin's solution and de-
calcified with 5% nitric acid. Upon being decalcified sufficiently, the resulting
temporal bones were dehydrated with ethylalcohol according to an ordinary method,
substituted for xylene and embedded in paraffin. The embedded tissues were cut 4 Din
thickness using a microtome and then subjected to hematoxylin-eosin and Gomori's
trichrome staining.

As a result of observation after one week, it was confirmed that, in the defect sections of the negative control group, the proliferations of fibrous connective tissues, activated fibroblast cells and vascular endothelial cells were observed and inflammatory cells were diffused. On the other hand, in the defect sections of the experimental group, the proliferations of the fibrous connective tissues and new blood vessels were observed. Moreover, it was observed that the inflammatory cells infiltrated through the environs of the grafted materials and fibrous cells appeared in the grafted materials.

Furthermore, the experimental results depicted in Fig. 5 were as follows. In the defect sections of the negative control group, no inflammatory cells were found, the fibrous connective tissues were filled generally therein, and capillaries were distributed widely in the connective tissues. It was seen that the new bone formations were increased on the edges of the defect sections and, even though few and far between, the osteoid tissue was extended from the edge of the defect section to the central section. On the other hand, it was confirmed in the defect sections of the experimental group that collagen fibers were filled therein and new capillaries proliferated in the vicinity of the grafted materials and in the connective tissues. It was observed that the new bone formations were increased similarly to the control group on the edges of the defect sections and osteoblast cells were divided, osteoid tissues and immature bones were formed in the grafted materials. Moreover, it was confirmed that the amounts of inflammatory cells were sharply increased in the vicinity of the grafted materials and the new bone formations were increased in the area contacting with the grafted material. Furthermore, it was seen that the new bone, the grafted material and the connective tissues were present all together with the regions where the connective tissues survived were shown between the new bone and the grafted material.

Experimental Example 4: Ames test

The following Ames test was carried out to measure the possibilities of causing a genetic toxicity by the β-TCP spherical granules of the present invention.

The Ames test was carried out as standard plate incorporation test with ISO 10993-3 having a relatively high detection sensibility for the genetic toxicity.

Salmonella strains (TA98, TA100 and TA102, purchased from WOO JUNG BSC, Inc.) were inoculated in nutrient broths and cultured at 37 °C for about 15 hours. After adding 0.1 Dof the respective cultured solutions of the strains, 0.1 Dof 0.5 g/D solution of β-TCP granule and phosphate buffered solution (or 0.5 Dof S-9 mixture in case of metabolic activation) to glass test tubes (13x100 D. Corning), 2 Dof top agar was added to be mixed. Here, the 0.5 g/D solution of β-TCP granule was prepared in such a manner
that 4 g of β-TCP granules were added to 20 D of saline solution (0.9% NaCl solution), the resulting solution was left as it was at 37 °C for 72 hours and then filtered using 0.8 Dsyringe filter. After culturing the mixed solution smeared on agar plates in an incubator at 37 °C for 72 hours, revertant colony counts were measured. The results were shown as F values, the ratio of an average value of test material to an average value of negative control group. The positive groups were arranged as the first group where TA98 was treated with dexon, a mutagenic substance and the second group where TA100 was treated with sodium azide.

The results were depicted in Table 4.

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<tr>
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<th>F value of TA98</th>
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<tr>
<td>TCP effluent (-S9)</td>
<td>1.07</td>
<td>1.17</td>
</tr>
<tr>
<td>TCP effluent (+S9)</td>
<td>0.95</td>
<td>1.38</td>
</tr>
<tr>
<td>Positive control group (-S9)</td>
<td>13.96</td>
<td>5.48</td>
</tr>
<tr>
<td>Positive control group (+S9)</td>
<td>10.04</td>
<td>4.86</td>
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As shown in Table 4, it could be learned that the β-TCP of the present invention was safe against the genetic toxicity according as the F values of the β-TCP of the present invention showed more than 2 in the positive control groups and the F values of TCP effluents showed less than 2 in all groups.
Claims

[1] A preparation method of porous β-tricalcium phosphate granules comprising the steps of:
arranging a gelatin solution by admixing water and gelatin powders;
arranging a tricalcium phosphate (TCP) slurry by admixing TCP precursor powders and porous precursors and adding the gelatin solution thereto;
forming spherical granules by adding the mixed TCP slurry to a dispersion medium being stirred and gelating the granules;
separating and washing the gelated spherical granule surfaces with an organic solvent; and
calcining and sintering the washed spherical granules to remove additives other than the TCP.

[2] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1,
wherein the gelatin solution is prepared by adding 6-20 parts by weight to 100 D of water.

[3] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1,
wherein the porous precursor used is a hollow spherical macromolecule, naphthalene, starch or their mixture.

[4] The preparation method of porous β-tricalcium phosphate granules as recited in claim 3,
Wherein the porous precursor used is a hollow spherical macromolecule powder of vinylidene chloride/acrylonitrile (PVDC) copolymer having a density below 0.1 g/D, an average particle size of 60-90 D and an external wall thickness of 0.5-1.5 D.

[5] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1,
wherein 1-5 parts by weight of the porous precursors are mixed with 100 g of TCP precursor powders.

[6] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1,
wherein 12-17 parts by weight of the mixture of the TCP precursor powders and the porous precursors are added to 100 D of gelatin solution.

[7] The preparation method of porous β-tricalcium phosphate granules as recited in claim 5,
wherein the mixture of the TCP precursor powders and the porous precursors and
the gelatin solution is stirred at 200 to 600 rpm.

[8] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1, wherein the dispersion medium is mineral oil or corn oil.

[9] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1, wherein the TCP slurry is firstly added to corn oil of room temperature and secondly added to corn oil kept in cold storage.

[10] The preparation method of porous β-tricalcium phosphate granules as recited in claim 9, wherein the first corn oil is added 1 to 3 times in volume more than the TCP slurry and the second corn oil kept in cold storage is added to 4 to 5 times in volume more than the TCP slurry.

[11] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1, wherein the organic solvent is chloroform, ether, hexane, ethanol, acetone or their mixed solvent.

[12] The preparation method of porous β-tricalcium phosphate granules as recited in claim 1, wherein the calcining and sintering step comprises the step of heat-treating the spherical granules at 1,000 to 1,180 °C under a heating rate of 0.15 to 1.10 °C/min to cause phase transition of the TCP precursors to β-TCP.

[13] The preparation method of porous β-tricalcium phosphate granules as recited in claim 12, wherein, prior to the heat treatment at 1,000 to 1,180 °C, the method further comprises the steps of:
removing moisture at 30 to 50 °C;
removing moisture and organic solvent at 250 to 350 °C; and
removing residual organic components at 570 to 670 °C.

[14] The preparation method of porous β-tricalcium phosphate granules as recited in claim 13, wherein the step of removing moisture at 30 to 50 °C is kept for 3 to 5 hours.

[15] The preparation method of porous β-tricalcium phosphate granules as recited in claim 13, wherein the step of removing moisture and organic solvent at 250 to 350 °C is kept at a heating rate of 0.15 to 1.10 °C/min for 2 to 4 hours.

[16] The preparation method of porous β-tricalcium phosphate granules as recited in claim 13,
wherein the step of removing residual organic components at 570 to 670°C is kept at a heating rate of 0.15 to 1.10°C/min for 4 to 6 hours.

[17] The preparation method of porous β-tricalcium phosphate granules as recited in claim 12, wherein the step of heat-treating the spherical granules at 1,000 to 1,180 °C is kept for 11 to 13 hours.

[18] The preparation method of porous β-tricalcium phosphate granules as recited in any one of claims 1 to 17, wherein the porous β-TCP granule has a diameter of 1.4-2.0 d, 1.0-0.6 d, 0.6-0.4 d, or 0.4-0.25 d, the diameter being appropriate to be used as a graft material.

[19] A bone graft material or a bone scaffold comprising porous β-tricalcium phosphate granules prepared according to the preparation method in any one of claims 1 to 17.
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DRAWINGS

[Fig. 1]

[Fig. 2]
A. CLASSIFICATION OF SUBJECT MATTER

**A61L 27/12(2006.01)**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

- A61L 27/00, A61L 27/12, A61L 27/56

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

- KR, JP IPC8 A61L 27/00, A61L 27/12, A61L 27/56

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

eKIPASS (KIPO interenal) "mineral oil", "tetracalcium phosphate", and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 03/082365 A1(RIZZOLI et al CH, Oct 9, 2003) —See the abstract</td>
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<td>A</td>
<td>US 5422340 A (ARTHUR et al Jun 6, 1995) —See the abstract</td>
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<td>Y</td>
<td>Novel magnetic microsphere on the basis of poly(vinyl acetal) as affinity medium for quantiative detection of glycated haemoglobin Journal of Chromatography Vol 711, pages 53-60, 1995 —See the abstract</td>
<td>1-19</td>
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Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier application or patent but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

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

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

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

& document member of the same patent family

Date of the actual completion of the international search

20 NOVEMBER 2006 (20.11.2006)

Date of mailing of the international search report

21 NOVEMBER 2006 (21.11.2006)

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