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□赴國外出差或研習心得報告一份

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☑出席國際學術會議心得報告及發表之論文各一份

☑國際合作研究計畫國外研究報告書一份

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Bone Tissue Engineering (1/3)

Abstract

In this paper, mixture of ammonium bicarbonate (NH$_4$HCO$_3$) and sodium chloride (NaCl) salt particulates was used as a porogen additive to fabricate macroporous biodegradable poly(DL-lactide-co-glycolide) (PLGA) scaffolds. A two-step salts leaching process was proceeded after the samples became semi-solidified. The processing time of this approach reduces from several days to half day comparing to the standard solvent casting/particulate leaching (SC/PL) technique. In addition, the mixture of polymer/salts/solvent can be easily handled and molded into any specific shape and geometry, such as thin sheet, cylinder, or bone-shaped scaffolds for special applications in tissue engineering. The results demonstrate that the scaffolds not only have well interconnected, open pore structure, but also have greater mechanical properties than those made from the standard SC/PL technique. Primary rat osteoblasts seeded into the scaffolds exhibited good biocompatibility. The method developed in this paper can be a promising approach to fabricate polymer scaffolds for tissue engineering applications.

Keywords: Tissue engineering, Scaffolds, Biodegradable polymers, Cell attachment, poly(poly(DL-lactide-co-glycolide))

Introduction

Bone defects, which result from tumors, disease and infections, trauma, and abnormal skeletal development, pose a significant health problem. The traditional biological methods such as autografts, allografts, and nondegradable materials were employed to manage the bone-defect problems. While all of these therapies have shortcomings themselves, tissue engineering offers a promising new approach to bone repair and overcomes the limitations of these conventional therapies. In this approach, a three-dimensional porous, biodegradable polymer scaffold that seeds the cells and serves as a template for tissue regeneration is necessary. Among the biodegradable polymers, poly(α-hydroxy esters) such as poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) are widely being investigated as materials to regenerate the bone tissues. These biodegradable materials are not only biocompatible with host tissues but also aid in the differentiation and proliferation of the desired cell types. They can be easily and reproducibly fabricated into desired geometry of porous scaffolds that degrade to low molecular weight compounds as the need for an artificial support diminishes. Pore morphology, porosity, as well as mechanical characteristics of the polymer scaffolds are important properties that need to be tailored to the type of tissue being regenerated.

Porous scaffolds have been prepared by numerous techniques including phase separation [1], fiber extrusion and fabric forming processing [2], gas foaming [3], and solvent casting/particulate leaching (SC/PL) [4]. The pore size of the biodegradable scaffolds prepared by phase separation is in the range of 1-20 μm, which is only amenable for fibroblast in-growth. This limitation was improved by inducing the coarsening effect in the phase separation process to enlarge the pore diameter (well above 100 μm) of the scaffolds. The fiber extrusion and fabric forming processing is popularly used for soft tissue engineering, but its mechanical strength is weak and not suitable for hard tissue regeneration. The scaffold fabricated by gas foaming often exhibits closed pore structure with a solid skin of polymer results on the exterior surface of the foamed matrix, which is disadvantageous in many
applications of cell transplantation. The limitation was improved by combining gas foaming and particulate leaching techniques to fabricate matrices with a well controlled porosity and pore structure. The SC/PL technique has been extensively used to fabricate macroporous scaffolds. In this method, salt particles are dispersed in a polymer solution, the solvent in which the polymer is dissolved is evaporated to produce a polymer/salt composite membrane. Salt particles are then leached out of the membrane by immersing the membrane in water. However, scaffolds prepared from this method often exhibit a dense surface skin with poor interconnectivity between macropores, which interferes with in vitro cell seeding as well as in vivo implantation. Additionally, it is found that the salts (NaCl) incorporated inside the polymer matrix are difficult to be leached out, it often takes several days to completely leach the salts out. Recently, a novel fabrication method of macroporous biodegradable polymer scaffolds was developed using gas foaming salt (NH₄HCO₃) as a porogen additive [5]. This results in the expansion of pores within the polymer matrix to a greater extent, leading to well interconnected macroporous scaffolds. However, in this approach if the volatile organic solvent in the solidified blend mixture is completely removed before proceeding to the gas foaming process, a dense polymer structure is often observed on the scaffold surface.

An alternate approach based on the SC/PL technique was developed from our research team. In this approach, the mixture of ammonium bicarbonate and sodium chloride salt particulates was used as a porogen additive to prepare PLGA scaffolds. Both ammonium bicarbonate and sodium chloride salt particulates are non-toxic and can be completely removed from the polymer matrix through a two-step salts leaching process after the samples become semi-solidified. Polymer scaffolds fabricated from this method demonstrate a highly open and interconnected macroporous structure. The current investigation focuses on effects of weight ratio of NH₄HCO₃/NaCl, weight ratio of salts/PLGA, and salts size ranges on the morphology, porosity, as well as mechanical properties of the scaffolds. Primary rat osteoblasts were seeded within the scaffolds to examine the biocompatibility.

**Experimental**

**Materials**
Poly(DL-lactide-co-glycolide) (PLGA, 75:25) was purchased from Aldrich (Milwaukee, WI) with an average molecular weight between 75,000-120,000. Chloroform (CHCl₃) was supplied by Riedel-deHaën (Seelze, Germany). Ammonium bicarbonate (NH₄HCO₃) and sodium chloride (NaCl) were obtained from Sigma (St. Louis, MO) and J. T. Baker (Phillipsburg, NJ), respectively.

**Scaffolds preparation**
Viscous polymer solution with a concentration of 0.1 g/mL was prepared by dissolving PLGA polymer in chloroform. Different weight ratios of NH₄HCO₃/NaCl sieved salt particulates (with particle size in the range of 150-710 µm) were added to the PLGA solution and mixed thoroughly with a glass bar. The weight ratio of salt particulates to polymer used was 7.3:1, 9:1, and 11.5:1, respectively. Table 1 lists the preparation conditions for the PLGA scaffolds. The paste mixture of polymer/salt/solvent was then cast into a special device equipped with a piece of glass slide (1 x 3 in²) as a sheet mold. The sheet (approximate 1 mm thick) was demolded after air-dried under atmospheric pressure for 1.5 hrs and then followed by vacuum drying for 1 hr (sample A3 excepted). A two-step salts leaching was proceeded after the sheet became semi-solidified. The sheet was first immersed in 90°C hot water (approximate 2 mins) to leach out the NH₄HCO₃ particles with concomitantly generating gaseous ammonia and carbon dioxide within a solidifying polymer matrix. After no gas bubbles were generated, the sheet was subsequently immersed into another beaker containing 60°C water (approximate 4 mins) to leach the rest of NaCl particles out. For sample A1, the sheet was first immersed in 90°C hot water for 1 min and subsequently immersed into another beaker containing 60°C water (approximate 4 mins) to leach the rest of NaCl particles out. The sample was then dipped into water (25°C) immediately for 1 min, and then freeze-dried for several days and stored in desiccator until use.

**Scaffolds characterization**
Differential scanning calorimetry (DSC, TA Instruments 2010) was employed to measure the glass transition temperature (Tg) of the prepared scaffolds. The specimen was scanned from 25 to 200°C with a heating rate of 10°C/min. The porosity of polymer scaffolds was determined by gross weight and volume measurements of the specimens after processing. To prepare tensile specimens, polymer scaffolds were cut into a square with a width of 1 cm and a length of 1 cm. Both ends of tensile specimen were clip into a special device attaching to the gripper. The tests were conducted on the tensile machine (Shimadzu, AGS-100D) with a cross head speed of 1 mm/min at room temperature. The mechanical properties (tensile strength, elongation at break, and tensile modulus) were taken from the average of ten specimens. Scanning electron microscopy (SEM, Jeol) was employed to view the pore morphology of the polymer scaffolds. The surfaces of specimens were prepared and shadowed with gold before investigation.

**Isolation of Osteoblastic stromal cells**
Osteoblastic stromal cells were isolated from calvariae of 1-3 day old Sprague-Dawley rats by a
sequential enzymatic digestion as described elsewhere [6]. Cells were collected from the third and fourth digests. The phenotype and function of the cells were characterized by the presence of alkaline phosphatase and deposition of calcium phosphate mineral in vitro. Osteoblastic stromal cell were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and maintained in 37°C, humidified, 5% CO₂/balance air incubator. Subculturing was performed using a buffered 0.05% trypsin solution. Cell subcultures between the second and third passage were used in all the experiments.

**Cell attachment and Histology**

The sterilized scaffolds (1 cm²) were placed in 24 well culture plates. These tissue culture plates coated with 12% poly-HEMA (hydroxyethylmethacrylate) held the scaffolds to ensure that the osteoblasts wound grow on only the biomaterials and not the tissue culture wells. β-counting method was used to examine the ability of cells attaching to the scaffold. The cells were incubated with medium containing 0.5 μCi/mL ³H-thymidine for one day before being seeded into scaffolds. The suspended cells (100 μL) incorporated with ³H-thymidine were seeded onto the scaffold and non-treated well, respectively, at a density of 5x10⁵ cells/mL. After being seeded 24 hours, the cell/polymer and cell/well were washed twice with phosphate-buffered saline solution, followed by incubating in 1N NaOH for 1 hour to disrupt the cell membrane. The samples were neutralized with 1N HCl and then 500 μL aliquots mixed with 3.5 mL counting fluid was transferred into counting vial (Ready safe, Beckman). The radioactivity of attached cells was measured by a liquid scintillation counter.

Besides, the osteoblast cells were seeded onto the scaffold at a density of 5x10⁵ cells/scaffold and incubated in growth medium supplemented with 10% fetal bovine serum, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate. The culture medium was changed every two days during culture. After two weeks, the cell culture was stopped and fixed for H&E stain.

**Results and Discussion**

Several above-mentioned methods have been employed to manufacture the polymer scaffolds. These methods have their advantages but also have their limitations. To improve some of these limitations, an alternate method was developed in this paper using mixture of NH₄HCO₃/NaCl particles as a porogen additive to fabricate polymer scaffolds. In this approach, viscous polymer solution was prepared by dissolving PLGA polymer in chloroform. The sieved NH₄HCO₃/NaCl salt particulates were added to the PLGA solution and mixed thoroughly to obtain a paste of polymer/salt/solvent mixture. The past mixture was demolded after air-dried and then followed by vacuum drying. A two-step salts leaching was proceeded after the sheet became semi-solidified to remove the salt particulates. The sheet was first immersed in hot water to leach out the NH₄HCO₃ particles with concomitantly generating gaseous ammonia and carbon dioxide within a solidifying polymer matrix. After no gas bubbles were generated, the sheet was subsequently immersed into another beaker containing warm water to leach the rest of NaCl particles out. The glass transition temperature of the prepared PLGA scaffold measured from differential scanning calorimetry (DSC) was found to be 55.0 °C, which was very close to that of raw material (53.1 °C). The thermal property demonstrated that the PLGA structure was not changed during the scaffold fabrication process.

Figure 1 shows the typical surface morphology of the PLGA scaffolds prepared by incorporation of 50 wt% of NH₄HCO₃ and 50 wt% of NaCl sieved salt particulates into the polymer solution (sample A2). The resultant scaffolds have a well-interconnected macroporous structure of pore sizes ranging from 300-500 μm, which is appeared to be dependent on the sizes of sieved NH₄HCO₃/NaCl particulates. The surface structures of scaffold are highly porous and the pores are uniformly distributed from the surface to the core region. Porosity for various scaffolds was determined by gross weight and volume measurements and listed in Table 2. Porosities increased with weight ratio of salts to polymer, as expected, and all the values were near or above 90%. These structures are often desirable in tissue engineering applications, which enable the transplanted cells and cells present in the host tissue to interact freely. From sample preparation, it was found that adding sodium chloride in ammonium bicarbonate salt particulates into the polymer gel solution prevented the rapid evaporation of chloroform from the blend mixture. This helps in retaining the solidified blend mixture in an elastic and rubbery state, which is more readily for gas foaming process to occur. In turn, the gas evolution of ammonium bicarbonate salt particulates occurs immediately and the gas flow-out rapidly throughout the semi-solidified matrix to the aqueous medium. This approach has two advantages over the standard SC/PL technique. First, the dispersion of mixture of NH₄HCO₃/NaCl particles in the polymer solution retained the polymer in an elastic and rubbery state rather than in a glassy state after air- and vacuum-dried, which prevents surface from forming dense structure. Second, the first step of leaching creates large pores inside the structure, which facilitates water flows-in throughout the polymer matrix and helps in removing NaCl particles. Accordingly, the processing time reduces from several days to half day comparing to the standard SC/PL technique. In addition, the mixture of polymer/salts/solvent can be easily handled and molded into any specific shape and geometry, such as thin sheet, cylinder (as shown in Figure 2), or bone-shaped (as shown in Figure 3) scaffolds, which can be fitted into the three-dimensional geometry of
specific tissue defects for special applications in tissue engineering.

In order to compare the surface morphology of polymer scaffolds prepared from different weight ratios of salts, ammonium bicarbonate and sodium chloride salt particulates were added along into the polymer gel solution, respectively, to prepare the polymer scaffolds. Figure 4 shows the surface morphology of PLGA scaffold prepared by adding ammonium bicarbonate salt particulates alone into the polymer solution (sample A3). Although all of the ammonium bicarbonate salt particulates were removed from the PLGA matrix through generating gaseous ammonia and carbon dioxide within the solidifying polymer matrix, open and partially closed macropores structures were present in the interior region, while a dense skin layer was always observed on the surface. The formation of dense skin layer was due to complete evaporation of the organic solvent from the interior region of the matrix to the surface and a subsequent increase in the polymer concentration of the remaining solution entrapmed within the salt bed. The carbon dioxide evolved from ammonium bicarbonate salt particulates may also act like a drying agent and extract organic solvent from polymer matrix. The closed pores and nonporous surface of the scaffold may prevent cells from migrating between the interior and exterior of the matrix, which is undesirable in tissue engineering applications.

In this study, polymer scaffold fabricated from mixture of PLGA/sodium chloride/solvent was also prepared (sample A1). From sample preparation, it was found that the addition of sodium chloride into the polymer solution made the mixture more viscous than that of PLGA/ammonium bicarbonate/solvent mixture prior subjected it to air and vacuum drying. This prevents the organic solvent being completely evaporated from the polymer matrix after air and vacuum drying and retains the polymer in an elastic and rubbery gel state before proceeding to the leaching process. The surface morphology of PLGA scaffold (Figure 5) shows clearly that all of the sodium chloride salt particulates were completely removed from the PLGA matrix under these leaching conditions. Highly open and interconnected macropores were uniformly distributed from the interior region to the surface of the PLGA scaffold. However, the very high degree of hydration between sodium chloride and leaching medium (water) causes the amorphous polymer being attacked by the water molecules during the leaching process. This results in a brittle nature scaffold with thinner cell struts distributed throughout the structure as shown in Figure 5, as compared to the struts shown in Figures 1 and 4. Although the scaffold keeps its structural integrity, the weak mechanical properties (data shown below) may limit its applications in tissue engineering.

Tables 3 to 5 list the mechanical properties of polymer scaffolds fabricated from different weight ratios of NH₄HCO₃/NaCl, weight ratio of salts/polymer, and salts particle size, respectively. The tensile strengths of polymer scaffolds made from this approach (ranging from 158 ± 60 to 390 ± 46 KPa) were about two to six fold greater than those manufactured from SC/PL technique (as compared to 95% porous PLGA scaffolds fabricated with NaCl salt sizes in the range of 106-425 μm, or greater than 425 μm). The values of tensile modulus of PLGA scaffolds made from this technique (ranging from 6.2 ± 2.4 to 113.1 ± 48.3 MPa) were significantly greater than that made from SC/PL technique (as compared to 95% porous PLGA scaffold fabricated from NaCl particles with a tensile modulus of 334 ± 52 KPa). The values of elongation at break for all the prepared scaffolds were also comparable to those made from SC/PL technique. The mechanical properties of PLGA scaffolds fabricated from different weight ratios of NH₄HCO₃/NaCl are listed in Table 3. From sample preparation, it was found that the scaffolds made from ammonium bicarbonate were stiffer than those made from sodium chloride particles. As a result, both tensile strength and tensile modulus of scaffolds made from ammonium bicarbonate were superior to those made from sodium chloride particles. This result was confirmed by comparing the surface morphology of PLGA scaffolds fabricated from different type of salts. As shown in Figures 4 and 5, the cell struts present in the PLGA scaffold made from ammonium bicarbonate were thicker than those fabricated from sodium chloride particles. The thickness of cell struts directly reflects the mechanical properties of the material, the thicker the cell strut, the greater the mechanical properties. The values of tensile strength and tensile modulus of scaffold made from 50 wt% NH₄HCO₃/50 wt% NaCl fall in between those made from ammonium bicarbonate and sodium chloride particles, as expected. Since a dense structure was always observed on the surface of the scaffold made from ammonium bicarbonate particles, it has the highest percent elongation at break among the others. The increase in porosity decreased the tensile strength, percent elongation at break, and tensile modulus of polymer scaffolds, as expected (shown in Table 4). The smaller particle size of ammonium bicarbonate results in smaller pore size inside the structure of the polymer scaffold, which has better ability to distribute the tensile force than the larger one. As a result, the tensile strength, percent elongation at break, and tensile modulus of the polymer scaffold decreased with increasing particle size of ammonium bicarbonate as indicated in Table 5.

Osteogenic differentiation was induced by culturing calvariae stromal cell in osteogenic medium (OM) (consisting of DMEM supplemented with 10% FBS, 10mM Na₃-β-glycerol phosphate, and 50 μg/mL L-ascorbic acid). In order to confirm their osteogenic capacity, the alkaline phosphatase (AP) activity and ECM calcification were examined. To detect AP activity, cells were incubated in OM for one week, rinsed with PBS, and stained with alkaline phosphatase kit (Sigma 851--2). For ECM calcification detection, the cells were incubated in OM.
for four weeks and stained with von Kossa. Stromal cells cultured in OM for 7 days formed an extensive network of dense, multilayered nodules that stained positively for AP as shown in Figure 6. After seeding four weeks, several black regions (calcified ECM) were observed in stromal cell treated with von Kossa stain. In sum, stromal cell culturing in OM can be induced osteogenic lineage.

Cell attachment for three dimensional scaffolds was determined by \( \beta \)-counting method. The cell attachment (PLGA) after 24 hours in culture was greater than 75% (Well was regarded as 100%) (see Fig. 7). Thus, PLGA scaffold demonstrates high cell attachment, which can prevent cells from losing after seeding. However, it still has long distance to go to achieve 100% attachment. In future, we will use nature biomaterials such as collagen, chitosan, and hydroxyapatite combined with PLGA to improve cell attachment.

**Conclusions**

PLGA scaffolds fabricated from mixture of ammonium bicarbonate and sodium chloride salt particulates not only have well interconnected, open pore structure, but also have greater mechanical properties than those made from standard SC/PL technique. Primary rat osteoblasts seeded into the scaffolds exhibited good biocompatibility. The method developed in this paper can be a promising approach to fabricate polymer scaffolds for tissue engineering applications.

**References**


**Table 1. Preparation conditions of PLGA scaffolds using chloroform as solvent**

<table>
<thead>
<tr>
<th>Sample</th>
<th>NH(_4)HCO(_3) (wt %)</th>
<th>NaCl (wt %)</th>
<th>Weight ratio of salts to PLGA</th>
<th>NH(_4)HCO(_3) size range ((\mu)m)</th>
<th>NaCl size range ((\mu)m)</th>
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</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
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<tr>
<td>A2</td>
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<td>50</td>
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<tr>
<td>A3</td>
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<td>0</td>
<td>9:1</td>
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<td>300-500</td>
</tr>
<tr>
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<td>50</td>
<td>50</td>
<td>7:3:1</td>
<td>150-300</td>
<td>300-500</td>
</tr>
<tr>
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<td>50</td>
<td>50</td>
<td>9:1</td>
<td>150-300</td>
<td>300-500</td>
</tr>
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<td>50</td>
<td>11:5:1</td>
<td>150-300</td>
<td>300-500</td>
</tr>
<tr>
<td>C1</td>
<td>50</td>
<td>50</td>
<td>9:1</td>
<td>150-300</td>
<td>300-500</td>
</tr>
<tr>
<td>C2</td>
<td>50</td>
<td>50</td>
<td>9:1</td>
<td>300-500</td>
<td>300-500</td>
</tr>
<tr>
<td>C3</td>
<td>50</td>
<td>50</td>
<td>9:1</td>
<td>500-710</td>
<td>300-500</td>
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**Table 2. Porosity of PLGA scaffolds made from different weight ratios of PLGA/salts**

<table>
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<tr>
<th>Sample number</th>
<th>Weight ratio of salts/PLGA</th>
<th>Porosity (%)</th>
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</thead>
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<tr>
<td>B1</td>
<td>7:3:1</td>
<td>89.42</td>
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<td>90.00</td>
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<tr>
<td>B3</td>
<td>11:5:1</td>
<td>91.09</td>
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</table>

**Table 3. Mechanical properties of PLGA scaffolds made from different weight ratios of NH\(_4\)HCO\(_3\)/NaCl**

<table>
<thead>
<tr>
<th>Sample number</th>
<th>NH(_4)HCO(_3) (wt %)</th>
<th>NaCl (wt %)</th>
<th>Tensile strength (KPa)</th>
<th>% Elongation at break</th>
<th>Tensile modulus (MPa)</th>
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<tr>
<td>A1</td>
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<td>10.3</td>
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<td>50</td>
<td>50</td>
<td>240</td>
<td>6.0</td>
<td>31.6</td>
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<td>100</td>
<td>0</td>
<td>390</td>
<td>11.1</td>
<td>113.1</td>
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</table>

**Table 4. Mechanical properties of PLGA scaffolds made from different weight ratios of salts/PLGA**

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Salt (parts)</th>
<th>PLGA (parts)</th>
<th>Tensile strength (KPa)</th>
<th>% Elongation at break</th>
<th>Tensile modulus (MPa)</th>
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<tr>
<td>B1</td>
<td>7.3</td>
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<tr>
<td>B3</td>
<td>11.5</td>
<td>1</td>
<td>242</td>
<td>5.2</td>
<td>27.7</td>
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Table 5. Mechanical properties of PLGA scaffolds made from different salt sizes

<table>
<thead>
<tr>
<th>Sample number</th>
<th>NH₄HCO₃ (μm)</th>
<th>NaCl (μm)</th>
<th>Tensile strength (KPa)</th>
<th>% Elongation at break</th>
<th>Tensile modulus (MPa)</th>
</tr>
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<tbody>
<tr>
<td>C1</td>
<td>150-300</td>
<td>300-500</td>
<td>257 (±44)</td>
<td>8.0 (±2.7)</td>
<td>35.3 (±10.1)</td>
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<tr>
<td>C2</td>
<td>300-500</td>
<td>300-500</td>
<td>240 (±49)</td>
<td>6.0 (±0.2)</td>
<td>31.6 (±11.2)</td>
</tr>
<tr>
<td>C3</td>
<td>500-710</td>
<td>300-500</td>
<td>158 (±60)</td>
<td>4.5 (±1.2)</td>
<td>25.9 (±16.8)</td>
</tr>
</tbody>
</table>

Figure 1. The typical surface morphology of the PLGA scaffolds prepared by incorporation of 50 wt% of NH₄HCO₃ and 50 wt% of NaCl sieved salt particulates into the polymer solution (sample A2).

Figure 2. Specific shape and geometry (thin sheet and cylinder) of PLGA scaffolds.

Figure 3. Bone-shaped PLGA scaffold.

Figure 4. The surface morphology of PLGA scaffold prepared by adding ammonium bicarbonate salt particulates alone into the polymer solution (sample A3).

Figure 5. The surface morphology of PLGA scaffold prepared by adding sodium chloride salt particulates alone into the polymer solution (sample A1).
Figure 6. Stromal cells induced with osteogenic medium (OM) express osteogenic ability. (a) Cells were stained after 1 week for AP activity (AP; blue) (100x) and (b) The calcified extracellular matrix (black regions) was examined after 4 weeks (von Kossa) (100x).

Figure 7. Cell attachment was determined by \( \beta \) -counting. The means of Wells is 1990 cpm (SD; 185) and PLGA is 1539 cpm (SD; 397). Each column is triplicate.

Figure 8. Cells/PLGA sectioned for H&E stain after seeding 24 hours.
出席國際會議報告

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The Biological Response of Human Osteoblasts on Plasma Sprayed Hydroxyapatite Coatings.

一、參加會議經過

於九十一年三月三十日由台北出發經桃園機場，搭乘長榮飛機往布里斯本，再轉機至阿德雷得，到達時機已為當地四月一日上午十點。趕到會場阿德雷得會議中心以觀察員身份參加 council meeting 會議。期間與會之我國代表為台大陳光光教授及長庚許文釵教授，會中另有中國入會議程，所以我們不敢掉以輕心。向來教授前主席和奧斯汀主席 Bose 溝通良好且我國參與人數眾多論文亦多，主席互動強烈，中方人員亦敢提出政治議題。四月一日報到與註冊後即同時將明年 2002 亞太產科會之海報及傳單，分享給與會人員。當日晚上參加大會同樂式，以各國小孩持名牌進場，為音樂節目以新潮裝扮之澳洲年輕人唱國歌 花為異國，四月二日起正式學術會議。本科林醫師第一天就報告。四月三日參加第二次 council meeting。本人兩篇皆在四月五日，第一篇為九點十五分報告論文“The Biological Response of Human Osteoblasts on Plasma Sprayed Hydroxyapatite Coatings。”第二篇為下午三點“Subvastus approach for TKR”。四月六日下午三點半到五點半為第十四屆大會之 council meeting，這次本人以正式國家代表身份與會，匆匆趕到機場搭乘六點半飛機到雪梨，飛行時間是晚上十一點。四月七日清晨飛到布里斯本轉機回台。

二、與會心得

本次與會除學術會議外，同時也是國家代表，參加會議，深感了解英文議事規則及會議記錄之重要，其次再結交為會議中提問支持的重要幫助。在學術議題上，以 metal on metal articulation 最獲得大家討論。正反兩方針針相對精采萬分，尤其充分利用 powerpoint 之動畫效果，印象深刻。第三是印度此次第一次與會，醫療資源不豐富地區有其處理疾病之獨到方針，其中以 Kelkar Procedure 最讓人印象深刻。小小手術可以改善末梢血液循環，不需要馬克氧不需 prostaglandinE，就大幅減少截肢機會，在國內應有應用之市場。

三、攜回資料名稱及內容

攜回本次大會論文集，共 234 頁。
The Biological Response of Human Osteoblasts on Plasma Sprayed Hydroxyapatite Coatings

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ABSTRACT

The purpose of this study was to investigate the biological responses of human osteoblasts on plasma sprayed HA coating (HAC), and the effects of surface roughness. In addition, the biological responses of human osteoblasts on HACs that pre-treated in the similar body fluid (SBF) were also investigated.

In this study, the HACs with 50μm thickness were made by atmosphere plasma spray (APS). The human osteoblasts derived from loose bone during the hip surgery were cultured in D-MEM with 4% FBS on polished HA coating (HACp), plasma sprayed HA coating (HAC) and polystyrene (PS)(as control). Part 1: The three specimens mentioned before were dipped in the SBF three days for pre-treatment (named t-PS, t-HACp, t-HAC). The cell adhesion and growth of pre-treated specimens were compared with non-treated specimens at the early phase (3, 12, 24 hours). Part 2: The cell growths (1, 3, 5, 10, 15 days) were evaluated by counting the cell number on the surface of PS, HAC and HACp. The biological functions of human osteoblasts were evaluated by the alkaline phosphatase activity (3, 5, 10, 15 days) and TGF-β concentration (5-15 days).

Part 1: At the early phase of cell culture (< 1 day), osteoblasts on pre-treated specimens surface showed the better attachment and growth than those culture on non-treated specimens. This result was due to the protein in the SBF provided the nutrient to cell, therefore, shortened the time of cell attachment and promoted the cell growth. Part 2: The cell morphology under SEM showed that cells on smooth PS and HACp surfaces had the better attachment than those on rough HAC surface. On cell growth and cell number (1, 3, 5, 10, 15 days), the PS was the best and HACp was better than HAC. It is due to the easy attachment and better growth on smooth surface than rough one. On alkaline phosphatase activity (APA), HAC showed better APA than HACp, the PS was the worst. On TGF-β concentration, the TGF-β secreted from human osteoblasts on rough HAC had higher concentration than smooth HACp and PS. The results mentioned above showed that the rough surface material promote the biological activity of human osteoblasts in late phase (5-15 days).

1. The HACs after pre-treatment in the SBF would promote the cell attachment on coating surface and speed the cell growth. 2. The results from this study showed that rough HAC expressed better biocompatibility than the smooth one and smooth HAC show early superiority of cell growth than rough one.
INTRODUCTION

In the application of biomaterial n orthopaedic surgery, bone cell response and biocompatibility of material play important roles in the bone-implant interface for long term survivor of the prosthetic implant fixation. The effect of the biocompatibility on bone formation is associated with the bone cell response induced by biomaterials. Recently, present studies use the model of cell culture to investigate the biological response of bone implant interface during early phase [1-3]. The factors of chemical composition, surface topography, surface energy and surface roughness of biomaterials would affect the bone cell response of implants, and further influence the biocompatibility in clinical use.

Some researches investigate the influence of surface surface roughness on bone integration of implants [4-6]. In vivo, studies report that rougher surfaces of titanium implant promote bone formation, whereas smoother surfaces tend to induce fibrous interfaces, and rough surface has higher mechanical strength in comparison with smooth surface [4-6]. Recently, some studies investigated the surface roughness of implants effect on bone cell response and how it examines with cell culture [7-12]. Most of these results suggest that rough implant surfaces alter osteoblast proliferation, differentiation and matrix production in vitro at later culture stage.

In orthopaedic surgery, Hydroxyapatite (HA) has the same chemical and crystallographic structure as the apatite of living bone, and can bond physicochemically with bone and promote bone growth onto its surface [13-16]. HA is therefore considered as an excellent bone substitute [17]. Among metals, increasingly widespread use of porous coating devices manufactured from titanium or its alloys is due to their relatively excellent corrosion resistance and favorable biocompatibility, compared to stainless steel and cobalt chromium alloys [18,19]. The combination of the mechanical properties of titanium and the biocompatibility of HA might provide implant device with good fixation to the surrounding bone. Clinical applications of HA-coating reported to promote the formation of normal bone at its surface [20-22]. Revealing excellent
biocompatibility and mechanical properties, plasma-sprayed bioactive HA-coated Ti-alloy implants have raised much interest as approach to achieve reliable implant to bone fixation.

Most studies reported that HA-coated Ti-6Al-4V implant is more osseointegrate and osteoconductive than uncoated ones in vivo [14,23]. In some studies, the cell growth and biological function of bioactive HA is superior than bioinert Ti-6Al-4V [24]. But, the study of Puleo et al. reported that less percentage cells were attached to the HA surface than to the titanium surface, and the doubling time of osteoblasts growth on HA surface was more than on the titanium at early cultured stage ($\leq 4$ days) [25]. The confused results express that the HA is not the absolutely superior in the stage of bone attachment and bone formation. In order to distinguish the influence of biomaterial on cell response, the stage from bone attached to bone formation needs further investigation.

In this study, four kinds of specimens, grit-blasted Ti-6Al-4V (Ti-b), polished Ti-6Al-4V (Ti-p), plasma-sprayed HA coatings (HAC), and polished HA coatings (HAC-p), were carefully prepared. The properties of Ti-6Al-4V and HA coatings were well identified to prevent the deviation during the preparation of specimens. The purpose of this investigation was to evaluate the influence of Ti-6Al-4V and HA coatings with different surface roughness on human osteoblast adhesion and morphologies during the early phase ($< 2$ days). Besides, the different contents of serum in cell culture medium were also adopted to investigate the cell attachment on the Ti-6Al-4V and HA with different surface roughness.
MATERIALS AND METHODS

Preparation of specimens

The 12.7mmΦ x 2.0mm disk plate used in this study was a surgical grade Ti-6Al-4V alloy (ASTM F136-92). The high-purity feed stock HA powder was adopted in the plasma-sprayed process, and the spraying parameter is listed in Table 1. For smoother surface of HA coatings, specimens were grounded by SiC papers and finally polished by 1.0 μm Al₂O₃ slurry. In the group of Ti-6Al-4V disks, the rough surface of Ti-6Al-4V was obtained by Al₂O₃ frit-blasted, and smooth surface of Ti-6Al-4V specimens was prepared by the following procedure: ground by SiC and finally polished by 1.0μm Al₂O₃ slurry. All the specimens were subjected to sonication five times in de-ionized water and one time in absolute alcohol and the packed in double-sealed autoclaving bags and steam sterilized at 121°C for 15 min.

Characterization of specimens

After surface and autoclaving treatments, the morphology of specimens was observed by scanning electron microscopy (SEM, Hitachi S-2500). The surface roughness of the specimens was quantified using a surface profilometer (Kosaka Lubarotary Ltd. Surfcorder SE-30H). The phase composition of HA coatings with or without polished treatment were analyzed by X-ray diffractometer (XRD, Rigaku D/Max III V) with a scan speed of 4° min⁻¹, using Cu Kα radiation (30 Kv, 20mA).

Shallower surface chemical analyses by X-ray photoelectron spectroscopy (XPS) were carried out for Ti-6Al-4V subjected to surface and autoclaving treatments. All analyses were carried out using VG Scientific ESCALAB 210 (analyzed area: 0.5 mm in diam.), operated at 12 KV and 20 mA in a pressure less than 10⁻⁸ mbar, using Mg Kα radiation. Measurements of binding energy in the range 0-1000Eev were made at a “take-off” angle 45° with respect to the sample surface. High-resolution scans of Ti, Al, V, and O peaks were performed on selected specimens.
Cell culture

Human osteoblasts were isolated from the loose bone during the hip joint surgical. Cells were collected from the third and fourth digestions, and phenotype and function of the cells were characterized by the presence of alkaline phosphatase and deposition of calcium phosphate mineral in vitro [26]. Osteoblasts were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and maintained in a humidified, 5% CO₂/balance air incubator at 37°C. Subcultured cells were used for experiments after two or three passages.

The sterilized disks were placed in 24-well culture plates. These tissue culture plates coated with 12% poly-HEMA (hydroxyethylmethacrylate) held the biomaterial samples to ensure that the osteoblasts would grow on the biomaterials only and not the tissue culture wells [27]. For experimentation purposes, the osteoblast cells were seeded on the disks at a density of 75000 cells/ml and flooded with growth medium supplemented with 4% fetal bovine serum (FBS), 50μg/ml ascorbic acid and 10 mM β-glycerophosphate. The culture medium was changed every two days during culture.

Cell number

The cell numbers on grit-blasted Ti-6Al-4V (Ti-b), polished Ti-6Al-4V (Ti-p), plasma-sprayed HA coatings (HAC), and polished HA coatings (HAC-p), were determined after 3, 6, and 24 hours of osteoblasts seeding. The two different contents (4% and 10%) of FBS were adopted to evaluate the effect of culture medium on cell number responses at the early phase. At designated times, the osteoblasts released from the biomaterial surface (by addition of 0.05% trypsin) contain 1 mM EDTA phosphate-buffered saline (PBS). The cell number was then counted using a hemacytometer.

Cell Morphology

Under culture medium containing 4% FBS, specimens (Ti-b, Ti-p, HAC,
and HAC-p) were prepared for SEM after 3-, 6-, 24- and 48-hours cultures. The medium was pipetted out from the dishes, and the plates rinsed several times with cacodylate buffer (pH 7.2), fixed for 2 hours with 2.5% glutaraldehyde (in 0.1M cacodylate buffered at pH 7.2); with post-fixation in 1% OsO₄ in buffer for 1 hour; dehydration in an ascending alcohol series; and immersed in HMDS (hemaethyldisilazane) for 10 min in lieu of critical point drying [28]. Finally, after sputter coating with gold, the specimens were examined using SEM at an accelerating voltage of 25 Kv.

Statistical analysis

Each data point represents the mean ± standard deviation of five individual cultures. The analysis of one-way variance (ANOVA) was used to evaluate the significance differences between cell growth and different kinds of materials. Differences were considered significant at $p \leq 0.05$. 
RESULTS

Characterization of specimens

Surface morphology

The SEM observation on the surfaces of Ti-p, Ti-b, HAC-p, and HAC is shown in Figure 1(a)-(d). After plasma spraying, HA was a complete molten matrix with accumulated splats, and the surface morphology had craggy shapes of peaks and valleys. In contrast, after polishing, HA coatings had a smoother surface with pores as found by the SEM observation. After grit-blasted treatment, surface morphology of Ti6Al4V (Ti-b) was characterized as irregular and rough topography in comparison with the smooth surface of Ti-p.

Surface roughness

The surface roughness of specimens is shown in Table 2. After polishing, the average surface roughness of Ti-p and HAC-p was significantly lower than Ti-b and HAC, and there was no significant difference found between Ti-p and HAC-p. As shown in Table 2, HAC was the roughest among all groups of specimens and statistically rougher than Ti-b.

XRD analysis

By XRD analysis, the phase composition of plasma-sprayed HA coatings consisted of HA phase and several impurity phases. These impurity phases included $\alpha$-Ca$_3$(PO$_4$)$_2$ ($\alpha$-TCP), $\beta$- Ca$_3$(PO$_4$)$_2$ ($\beta$-TCP), Ca$_4$P$_2$O$_9$, and CaO. After polishing, the phase composition of HAC-p was consistent with HAC specimens. The relative contents of phase composition of HAC and HAC-p were shown in Table 3. The results indicated that the polishing treatment has not changed the phase composition and contents in HA coatings.
XPS analysis

After autoclaving treatment, the broad-range XPS spectra from the nano-
surface of two different surface treatments for Ti6Al4V were shown in Figure 2.
The scans for the two surface treatments are very similar, with no indication of
the presence of V, while the presence of V on the alloy sample can be detected
by the V 2p3 peak at 515 eV on a more sensitive scale. High-resolution Ti 2p
spectra were examined to probe the changes in the nature of the oxide layers as
a function of surface treatment. The predominant oxide phase is TiO2, and the
various suboxide phases, Ti2+, Ti3+, and metallic Ti were also seen in the
spectra of Ti-b and Ti-p. The thickness of the oxide layer was estimated by
comparing peak intensity of the total Ti 2p3 oxide component to the Ti 2p3
metallic component. Using the formula devised by Lausmaa and Kasemo with
an electron mean free path of 1.7 nm [29], the surface oxide thickness for Ti-p
and Ti-b after autoclaving treatment were 2.25 nm and 2.34 nm, respectively.
In high-resolution scans of O 1s spectra, basic and acidic OH groups were
formed in the oxide surface of Ti-p and Ti-b by assuming chemisorption, and
their chemical nature arises from the way they bond, either to one or two
titanium ions. Table 4 summarizes the results of oxygen spectra for the two
different specimens with autoclaving treatment. As shown in Table 4, the
contents of basic and acidic groups were similar between Ti-p and Ti-b.

Cell attachment

After 3-, 6-, and 24-hour culture in medium containing with 4% FBS,
the cell number of osteobasts attached on the different specimens (Ti-p, Ti-b,
HAC-p, and HAC) were counted. Under culture medium with 4% FBS (Figure
3), the level of cell attachment to the surface of polished Ti6Al4V (Ti-p) was
significantly higher than Ti-b and HAC during all culture periods. However,
the statistical difference is only found between Ti-p and HAC-p only after 24-
hour culture. The osteoblast attachment to the smooth surface of HAC-p was also statistically higher than rough surface of HAC in all culture periods. During all experimental periods, the cell attachment on the surface of HAC-p was higher than Ti-b, while the statistical differences were not found in cell attachment for HAC-p and Ti-b. Between HAC and Ti-b specimens, the higher osteoblast adhesion was found at the surface of Ti-b than HAC, and the significant differences were found in all experiment periods.

Under 10% FBS, the graph of the osteoblast attachment versus time for the four kinds of specimens is illustrated in Figure 4. Increasing the content of FBS from 4% to 10% in medium, the higher level of osteoblast attachment was found on the surface of specimens under 10% serum, and significant differences were found between the same specimens cultured with two different contents of FBS in all experimental periods. Under culture medium with 10% FBS, the osteoblast attachment could be divided into three more levels based on three groups of specimens, (1) Ti-p and Ti-b, (2) HAC-p, and (3) HAC. During all culture periods, the level of osteoblast attachment to the surface of Ti-b and Ti-p is significantly higher than HAC-p and HAC, and there was no statistical difference between Ti-p and Ti-b. Between HAC-p and HAC, the significantly higher level of osteoblast attachment on HAC-p was also found than the one to HAC during all culture periods.

Cell morphology

In contrast to 10% serum condition, there were apparent differences found among four kinds of specimens in 4% FBS condition. Therefore, in this study, the osteoblast morphology was investigated under medium containing 4% FBS to evaluate the influence of surface roughness.

After 3 hours of culture, the osteoblast morphology on the surface of specimens was observed by SEM (Figure 5). On the smooth surface of Ti-p, the
cell morphology demonstrates rough texture, and the thin rim of cytoplasm had spread out from the cell body whose nucleus was clearly observed. The osteoblast morphology on HAC-p surface was similar to the one on Ti-p. However, the more elongated and less flattened morphology was observed on HAC-p. By the observation of SEM, the attached cells demonstrated spherical shape and protruded surface on the rougher surface of Ti-b and HAC.

After 6 hours of culture, Figure 6(a)-(d) represent the micrographs of osteoblasts on all four specimens, respectively. On the surface of Ti-p, the cells spread from the center, and the filopodia extended along the substrate surface (at the base of the cells with fibers observed in the extracellular matrix). The osteoblast morphology on HAC-p surface was similar to the one on Ti-p, however, the more elongated and less flattened morphology was observed on HAC-p. In comparison with smoother surfaces of HAC-p and Ti-p, the multiple filopodia extended and the cells appeared polygonal on the rougher surface of Ti-b and HAC.

After 24 hours of culture, the osteoblast morphology on the surface of specimens is shown in Figure 7. The cytoplasm was almost completely spread, and shows a flattened morphology with almost no dorsal ruffles and filopodia on the surface of Ti-p. The osteoblast morphology on the surface of HAC-p was similar to Ti-p, while the more elongated and less flattened morphology was still observed on HAC-p compared to the one on Ti-p. The presence of corroded surface was observed easily by the existence of crack in the surface of HAC-p. At 24-hour culture, the cell morphology on the rough surface of Ti-b and HAC were similar to that of 6-hour culture.

Figure 8(a)-(d) represent randomly taken SEM osteoblast on Ti-p, Ti-b, HAC-p, and HAC after 48 hours culture. On the surface of Ti-p, the osteoblast morphology at 48 hours was similar to 24 hours, and 48 hours exhibited a more confluent and a fibrous network between the cells. The osteoblast morphology
on the surface of HAC-p was similar to Ti-p at 48 hours. The edge of the spreading cytoplasm at the rougher surface of Ti-b and HAC was not readily distinguishable, and the cells on Ti-b and HAC had spread incompletely, while the cell structure was more compact. They exhibited more cubodial cell morphology, and appeared more elongated and less flattened. Some cells spanned the macropores apparently by first extending long filopodia across the pores.
DISCUSSION

In this study, the polishing treatment to produce two different roughness of HA coatings was adopted. In the previous studies [30,31], different roughness levels of surface could be manipulated by different plasma-sprayed parameters. By XRD analysis, it was found, moreover, different phase contents in HA coatings would be changed by different plasma sprayed parameters. For example, higher enthalpy parameter will obtain rougher surface with higher impurity phases in HA coatings (which would present the higher dissolution rate and more loosened structure in serum added simulated body fluid [30,31]). In vivo, the higher contents of impurity phase in HA coating, would easily induce dissociated particles from coatings and cause adverse biological responses in comparison with lower contents of impurities phase in HA coatings [23,24].

For Ti-6Al-4V, two kinds of surface treatment were used to obtain different surface roughness. There is a possibility that two different surface properties with different treatment, polishing or grit-blasting might be obtained. In clinical use, the reasons for the apparent success of titanium or its alloy implants have been attributed to the existence of a thin, stable passivating oxide layer of TiO2. Albrektsson et al. and Kasemo et al. mentioned that the biocompatibility of titanium implants is associated with the surface titanium oxide, not with the titanium metal [19,32]. After autoclaving treatment, the properties of titanium oxide on the surface of polished Ti-6Al-4V were similar to grit-blasted Ti-6Al-4V by XPS analysis. For example, the similar oxide thickness and contents of amphoteric OH groups were observed between two different surface treatment specimens. In this study, the behavior of osteoblasts at the Ti-p and Ti-b could be attributed to the factor of surface roughness.
Under 4% FBS condition, the levels of cell attachment on the smooth surface were significantly higher than the one on the rough surface of the same experimental materials. The result was consistent with the cell morphology. By the observation of SEM, the osteoblasts on the smoother surface exhibited a broadly spread and flattened morphology compared to cuboidal morphology on the rougher surface. In the studies of other researchers, they concluded that more flattened and well spread cells would show higher proliferate rates than those round spherical cells. For example, Folkman and Moscona and Archer et al. mentioned that one of the main regulators of proliferate rate in anchorage dependent cells is shape. In their studies, the cells, attached to materials with less spread, will show lower proliferate rates than those on the material with better spreading [33,34]. Similar results were also observed by Hunter et al [35]. They found that the surface of biomaterials with the greatest number and area of adhesion plaque spread well and flattened whilst those materials with the least number of adhesion plaque were more rounded and less spread.

When FBS contents was increased to 10%, no significant difference of cell attachment was found between Ti-p and Ti-b during all culture periods. The result indicates that the increasing FBS content diminishes the factor of Ti-6Al-4V surface roughness. As a general rule, cells do not directly bind to material surfaces rather than to extracellular glycoproteins on the surfaces of implants. After plating, the serum conditioned the reaction of material surface to provide better environment for cell attachment and spreading. In the study of Schneider et al. and Bagambisa et al.[36,37], some cell adhesion proteins in serum enhanced cell attachment and spreading. In this study, the higher serum content (10% FBS) diminished the factor of surface roughness on the osteoblasts attachment at Ti-6Al-4V, but the similar result was not found between HAC-p and HAC. As shown in Table 2, average surface roughness of HAC-p and HAC were 0.67 μm and 10.37 μm, respectively. The significantly
different levels of surface roughness between HAC-p and HAC should be the main reason for the reduction of influence of serum contents.

Of the two different experimental materials, Ti-6Al-4V (with polishing treatment) provided more optimal surface properties for osteoblast attachment than HA coating with the same polishing treatment. As shown in Figure 7(c), the surface of HAC-p was seriously attacked by surrounding medium, and cracks were also easily found after 24-hour culture. In the previous study, the corroded surface of HA coatings were well investigated in simulated body fluid containing serum[31]. The results showed that a bonding degradation of approximately 25-33% of the original strength was measured after HA coatings being immersed in SBF, and that predominantly depended on the period of immersion. The surface morphologies of HA coatings have dissolved in the SBF, and it is suggested that since the interlamellar structure of the HAC was weakened the bonding strength now becomes degraded. In this study, the loosened structure of HAC-p not can provide optimal surface for osteoblasts attachment compared to Ti-p. Because it is impossible to produce 100% denser HA coatings by plasma-sprayed technique, the porosity and pore in HAC-p is possible to be the another reason for the behavior of osteoblasts.

In this study, titanium has superiority of osteoblast adhesion than HA coatings during 2-day culture. The result is consistent with the studies of Pulco et al. and Santis et al.[12,25]. However, these results are different from those demonstrated clinically. In clinical use, the HA-coated Ti-6Al-4V implant is more osseointegrate and osteoconductive than uncoated ones[14,23]. In vivo and in vitro, the bone formation is a serial process, and the cell behavior is modified with serial changes of environment. Although the Ti-6Al-4V provided better surface for osteoblast adhesion during early phase, the effect of biomaterials on later frames of osteoblasts differentiation, matrix production, and calcification was as important as cell attachment and played a major role in
the long term implant success. However, in order to comprehend the effects of biomaterials and to obtain a complete interpretation of these events, further investigation of a series of experiments at different stages is required.
Conclusions

This work was carried out to investigate the effect of surface roughness, serum contents, and experimental materials on osteoblast attachment and spreading. By careful preparation, the phase composition and content of HA and surface properties of Ti-6Al-4V were unchanged after surface treatment. Under 4% FBS condition, the cell attachment at the smoother surface was significantly higher than rougher surface of same experimental materials. No statistical difference was found between polished and grit-blasted Ti-6Al-4V when the serum was increased by up to 10%. By SEM, the osteoblasts were early spreading and flattened at the smoother surface under 4% FBS, on the other hand, the cells were adhesive elongated and has a less flattened morphology at the rougher surface. During early periods (up to 48 hours), Ti-6Al-4V provided more optimal surface properties for osteoblasts attachment than HA coating after the polishing treatment, and this could be attributed to the loosen structure of HA coatings after immersion in culture medium.
REFERENCE

12. D. De Santis, C. Guerriero, P. F. Nocini, A. Ungersbock, G. Richards, P.
    601-607.


Table List

Table 1. Plasma spraying parameter employed for preparing the HA coatings.
Table 2. Average surface roughness (μm) of Ti-6Al-4V and HA coating (HA) with different surface treatment.
Table 3. The concentrations (in wt%) of the impurity phase of the as-sprayed and polished HA coatings.
Table 4. High resolution XPS surface chemical analyses (at%) of oxygen spectra for Ti-6Al-4V specimens with autoclaving treatment
Table 1. Plasma spraying parameter employed for preparing the HA coatings

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<td>Powder carrier gas, flow rate (l min⁻¹)</td>
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<td>Power (kW)</td>
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<td>Stand-off distance (cm)</td>
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<td>Surface speed (cm min⁻¹)</td>
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<tr>
<td>Transverse speed (cm min⁻¹)</td>
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</tbody>
</table>

Note: Plasma spraying was performed with a Plasma-Technik system (M-1100 C).
Table 2. Average surface roughness (µm) of Ti-6Al-4V and HA coating (HA) with different surface treatment.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Surface treatment</th>
<th>Roughness</th>
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<tbody>
<tr>
<td>Ti-6Al-4V</td>
<td>Polished</td>
<td>0.63 ± 0.11</td>
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<tr>
<td></td>
<td>Grit-blasted</td>
<td>4.64 ± 0.38</td>
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<tr>
<td>HA coating</td>
<td>Polished</td>
<td>0.67 ± 0.13</td>
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<tr>
<td></td>
<td>As-sprayed</td>
<td>10.37 ± 1.35</td>
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</table>

Note. The value is presented by mean ± standard deviation.
Table 3. The concentrations (in wt.%) of the impurity phase of the as-sprayed and polished HA coatings.

<table>
<thead>
<tr>
<th>Impurity phase</th>
<th>As-sprayed</th>
<th>Polished</th>
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<tbody>
<tr>
<td>α-TCP</td>
<td>16.38%</td>
<td>16.38%</td>
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<tr>
<td>β-TCP</td>
<td>12.77%</td>
<td>12.75%</td>
</tr>
<tr>
<td>Ca₄P₂O₉</td>
<td>15.23%</td>
<td>15.12%</td>
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<tr>
<td>CaO</td>
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<td>6.03%</td>
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</table>

Note: Data obtained by the internal standard method.
Table 4. High resolution XPS surface chemical analyses (at%) of oxygen spectra for Ti-6Al-4V specimens with autoclaving treatment

<table>
<thead>
<tr>
<th>Surface treatment</th>
<th>Ti-OH basic</th>
<th>OH acidic</th>
<th>O1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polished</td>
<td>10.76</td>
<td>21.66</td>
<td>67.58</td>
</tr>
<tr>
<td>Grit-blasted</td>
<td>11.09</td>
<td>20.98</td>
<td>67.93</td>
</tr>
</tbody>
</table>
Figure List

Figure 1. The surface morphologies of specimens: (a) polished Ti-6Al-4V (Ti-p), (b) grit-blasted Ti-6Al-4V (Ti-b), (c) polished HA coatings (HAC-p), and (d) as-sprayed HA coatings (HAC).

Figure 2. Broad-range XPS spectra of Ti-6Al-4V with autoclaving: (a) polished and (b) grit-blasted.

Figure 3. Under 4 % serum condition, the growth of osteoblasts on different specimens. Values are the mean ± standard deviation (n=5).

Figure 4. Under 10 % serum condition, the growth of osteoblasts on different specimens. Values are the mean ± standard deviation (n=5).

Figure 5. Under 4 % serum condition, Scanning electron micrographs of osteoblasts cultured for 3 hours on (a) polished Ti-6Al-4V (Ti-p), (b) grit-blasted Ti-6Al-4V (Ti-b), (c) polished HA coatings (HAC-p), and (d) as-sprayed HA coatings (HAC).

Figure 6. Under 4 % serum condition, Scanning electron micrographs of osteoblasts cultured for 6 hours on (a) polished Ti-6Al-4V (Ti-p), (b) grit-blasted Ti-6Al-4V (Ti-b), (c) polished HA coatings (HAC-p), and (d) as-sprayed HA coatings (HAC).

Figure 7. Under 4 % serum condition, Scanning electron micrographs of osteoblasts cultured for 24 hours on (a) polished Ti-6Al-4V (Ti-p), (b) grit-blasted Ti-6Al-4V (Ti-b), (c) polished HA coatings (HAC-p), and (d) as-sprayed HA coatings (HAC).

Figure 8. Under 4 % serum condition, Scanning electron micrographs of osteoblasts cultured for 48 hours on (a) polished Ti-6Al-4V (Ti-p), (b) grit-blasted Ti-6Al-4V (Ti-b), (c) polished HA coatings (HAC-p), and (d) as-sprayed HA coatings (HAC).