CHAPTER 2

Experimental

2.1 Materials and equipments

2.1.1 Materials

1) Calcium carbonate ($CaCO_3$), analytical grade, Ajax, Netherland.
2) Ammonium dihydrogen phosphate ($NH_4H_2PO_4$), analytical grade, Merck, Germany.

2.1.2 Equipments

1) Analytical balance, model EK– 610i, A&D, Tokyo, Japan.
2) Analytical balance, model PW 254, Adam Equipment, Danbury, U.S.A.
3) Automatic Vickers Microhardness Tester, model A041554, Future tech, Japan.
4) Atomic absorption spectroscopy (AAS), model Varian SpectrAA 220, Australia.
5) Differential thermal analyzer, model STA 409 EP, U.S.A.
6) Electric furnace, model 3504, Eurotherm, Ashburn, U.S.A.
7) Fourier transforms infrared spectrometer (FTIR), model spectrum 2000, PerkinElmer, Waltham, U.S.A.
8) Hydraulic compressing machine, Golden gear, Shanghai, China.
9) Milling jar, a nylon polyamide 6, Bangkok, Thailand.
10) Oven furnace, model OVM-320-010E, Memmert, Germany.
11) Particle size analyzer, model Mastersizer S, Malvern, England.
12) Planetary ball mill machine, Compound clay, Bangkok, Thailand.

13) Scanning electron microscope (SEM), model JSM-6335F, JEOL, Tokyo, Japan.
14) Surgical instrument kits for experimental animal, Kent scientific corporation, U.S.A.
15) Surgical Blades, No. 15, Swann morton Company, England.
16) Suture, Vicryl plus 2/0, Kent Scientific Corporation, U.S.A.
17) Universal mechanical testing machine, model LRX, Lloyds Instruments, Leicester, England.
18) X-ray diffractometer (XRD), Bruker, 8D Advance, England.
19) X-ray fluorescence (XRF), model Magix pro, PW2540 vrc sample changer, U.S.A.
20) Yttrium stabilized zirconia grinding media, cylindrical body, Inframat®, U.S.A.

2.2 The manufacturing of synthetic HA

2.2.1 Preparation of mixed powder

1) HA was synthesized by solid state reaction between $CaCO_3$ and $NH_4H_2PO_4$ powder. The mixed powders for each ratio were prepared for 100 g as followed in **Table 1.**

Table 1 Composition of $CaCO_3$ and $NH_4H_2PO_4$

Ca/P mole ratio	$CaCO_{3(s)}$ (g)	$NH_4H_2PO_{4(s)}$ (g)
1.65	58.93	41.07
1.66	59.09	40.91
1.67	59.20	40.80
1.68	59.36	40.64
1.69	59.50	40.50

Sample code: XY - Z

X = 1.65, 1.66, 1.67, 1.68, 1.69 used symbol = 1, 2, 3, 4, 5

Y= 1100 °C, 1150 °C, 1200 °C, 1250 °C, 1300 °C used symbol = A, B, C, D, E

Z = 2 h, 3 h, 4 h, 5 h used symbol = 2, 3, 4, 5

2) The starting powders were combined in appropriate quantities with a Ca/P mole ratio of 1.65, 1.66, 1.67, 1.68 and 1.69. The first $CaCO_3$ powder was calcined at 650 °C for 5h. The first step was weighed of $CaCO_3$ and $NH_4H_2PO_4$ powder in mortar and mixed using by stirred of pestle for approximately 3 to 5 minutes for deagglomerated of mixture powders. The second step was provided mixture powders into a nylon polyamide milling pot with yttrium stabilized cylindrical zirconia grinding media in diameter of 10 x 10 mm. The grinding media were used approximately 0.81 kg per pot. Then the pot was put in a planetary ball mill machine at approximately 500 rpm for 45 min. The milling process of mixture powder consists of: (a) grinding of mixture powders was ground for 15 min and was stopped for 5 min and next ground for 15 min and stopped for 5 min and finally step was ground for 15 min, and (b) subsequently, opened the cover of pot mill, followed take out of mixed powder and placed in a desiccators for removed moisture and cool down.

2.2.2 Forming of a green body

Mixture powders were prepared about 120 g for each Ca/P mole ratio. The green body was weighed about 1 g per piece. The obtained powder was pressed with an uniaxial pressing machine into disc shape with the dimension approximately of 4 mm in thickness and 16.5 mm in diameter under stresses approximately 3 MPa in cylindrical stainless steel die. The dye wall was lubricated using a liquid solution of 5 wt% stearic acid in ethanol. The green bodies had 120 samples from preparation.

2.2.3 Sintering of a green body

The green bodies of samples were sintered at temperature from 1100, 1150, 1200, 1250 and 1300 ˚C for 2, 3, 4 and 5 h in electric furnace. Sintering process was carried out by setting a heating ramp rate and soaking time as shown in **Figure 8**. Sintering was carried out at a heating ramp rate of 60°/hour up to 400 ˚C with a soaking time of 30 min, then a heating ramp rate of 120°/hour up to 650 ˚C with a soaking time of 1 hours. Then a heating ramp rate of 300°/hour up to isothermal sintered at different temperatures with a soaking time varied of 2 to 5 hours. Thereafter a heating ramp rate of 120°/hour down to 850 ˚C with a soaking time of 1 hours and finally furnace cooled down to room temperature with ramp rate of 240°/h. All sintered samples were then polish out to about 1 mm for smooth surface.

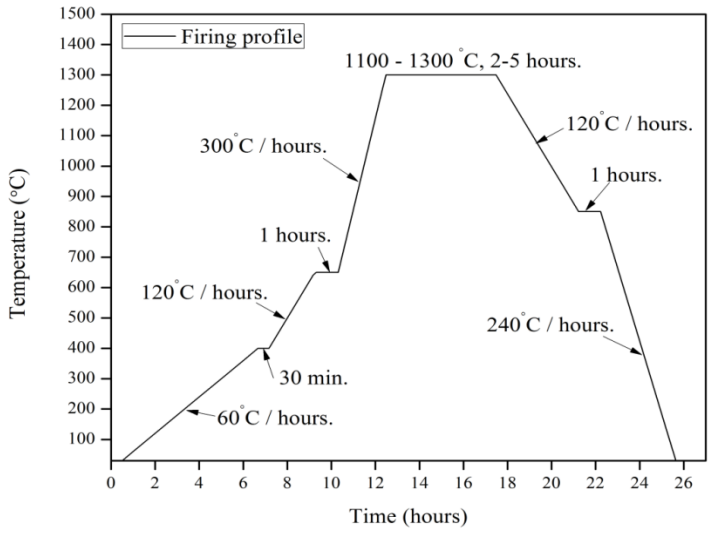

Figure 8 Firing profile of the sintering schedule of sintered samples.

2.2.4 Characterization of sintered samples

This research was characterized of raw materials powder, the powder samples after sintering of green body at various compositions, temperatures and soaking times.

1) $CaCO_3$ was characterized total heavy metals using AAS technique for confirmed safety in human body. The maximum allowable limit of all heavy metals determined as lead shall be 50 parts per million (ppm).

2) Multiple phases of all samples were analyzed using XRD in reflection mode with Cu-Kα (λ = 1.540598 Å) radiation. The data were analyzed in the 2θ range between 10º and 80º with a scanning step of 0.04º per minute. XRD analysis of powder shall be consistent with Joint Committee on Powder Diffraction Standards (JCPDS) file number 85-1108 for $CaCO_3$ [67]. $CaCO_3$ powder and the sintered sample were analyzed phase purity using XRD technique. It shall be consistent with JCPDS file number 09-432 for HA [68] and JCPDS file number 09-169 for TCP [69] and JCPDS file number 37-1497 for calcium oxide [70]. The HA phase content ratios were calculated using the relative intensity ratio (RIR) [71, 72]. One powder of sintered sample selected from six samples of each condition for analysis. They have been established XRD-based peak area calibration curve for samples of known HA and the other phase contents.

3) Process of particle size distribution composed of; (a) mixture powder before mixing and grinding (b) after mixing and grinding for 45 minutes. These powders were characterized particle size and the specific surface area by laser diffraction. Mixture powders were suspended in ethanol.

4) Thermal analysis was characterized by DTA/TG technique. The designed pattern of the heating schedule from DTA/TG curves of formation of HA phases. Mixture powders were used a Ca/P mole ratio of 1.67 amounts 200 mg for analysis and in the temperature range 40 to 1200 °C, rate 10 °C per minute for 2 hours in alumina crucible and alumina powder was used as the reference material.

5) The densities of sintered samples were measured by the Archimedes method using distilled water as medium. First, the bulk samples were dried in oven furnace at 110 °C for 4 hours, then measured dried weight as (D). Second, dried samples were boiled in distilled water for 4 hours and then continuous soaked for 12 hours. Third, bring the samples measured the weight of the water displaced as (I) and finally, take the samples of absorbed at the surface of the samples with tissue paper. Then, a measured the soaked weight in air as (S). These values are used in calculations the apparent density, bulk density, true density and apparent porosity.

These densities were determined from the equation (7) to (10) [73, 74]. The result was derived from six hundred samples of all conditions and the average value of each density calculated from six samples of each condition.

$$\text{Apparent density} = \left(\frac{D}{D-I}\right) \times \rho_{H2O} \quad (7)$$

$$\text{Bulk density} = \left(\frac{D}{S-I}\right) \times \rho_{H2O} \quad (8)$$

$$\text{True density} = \frac{(m_2-m_1)}{(X-Y)} \times \rho_{H2O} \quad (9)$$

$$\text{Apparent porosity} = \left(\frac{S-D}{S-I}\right) \times 100 \quad (10)$$

where, D = dried weight

I = the weight of the water displaced

S = soaked weight in air

ρ_{H2O} = density of distilled water at 21.5 °C was 0.997879 g/cm^3

True volume is solid component only. Exclude all the pores and body pieces ground into powder form and using a density bottle.

m_1 = weight of bottle and cover after dried

m_2 = weight of bottle, cover and solid powder

m_3 = weight of bottle, cover, distilled water and solid powder

m_4 = weight of bottle, cover and distilled water

(m_4 - m_1) = weight of distilled water required to fill the bottle (x)

(m_3 - m_2) = weight of distilled water required to fill the bottle above the solid powder (y)

x - y = the weight of distilled water occupying the same volume as the solid powder

6) Microhardness measurements were using a Vickers hardness tester and pyramidal diamond indenter used 50 g load for 15 second. Measurement of the lengths of the diagonals of indents of hardness and measured with an optical microscope. There was selected one sample from each condition (six samples) of which to provide measured hardness. Five indentations were made for each sample and the average value was taken calculated using the equation (11) [75].

$$HV = 0.1891 \frac{F}{\bar{d}^2} \qquad (11)$$

where, HV = Vickers hardness

F = Indentation load in Newton (N)

$\bar{d}$ = Mean diagonal length of indentation in micrometer (μm)

7) Bending strength was investigated using a ball-on-ring test using universal testing machine. This method was measuring biaxial strength for ceramic plates and thin ceramic disks. Ball -on-ring test was used a ball bearing for the support ring. The ball bearing minimum friction was between the sample and the support ring. Load was applied using a tungsten carbide ball that was 10 mm in diameter. The support rings were changeable and had diameters of 16 and 20 mm for the 20 mm and 25 mm diameter samples. A screw type universal testing machine was used to load cell in Newton to the piston of a ball-on-ring apparatus. The load was used with a crosshead speed of 0.254 mm/min (0.01 in/min). The loading rate was about 15 and 10 MPa/second (maximum stress) for the 20 mm and 25 mm samples [76].

The cross head was turned round immediately when failure appeared and to prevent crushing the fractured sample [76].

This research was adapted from above theory. Load cells were used 2500 Newton. The span length was 20 mm and the cross head speed was 0.5 mm/min. Six samples were tested and the average was appeared in all tests. The bending strength (σ) of the samples were determined from the equation (12).

$$\sigma = \frac{3F(1+\nu)}{4\pi t^2}\left[\frac{(1-\nu)}{(1+\nu)} \times \frac{2a^2 - b^2}{2R^2} + 2\ln\left(\frac{a}{b}\right) + 1\right] \quad (12)$$

where σ is the strength in MPa; F is the breaking load in Newton; ν the Poisson ratio; a is the radius of the support (mm); R is the radius of the samples (mm); t is the thickness of the sample (mm); and b is the t/3 (mm).

8) The microstructure of the sintered sample was studied by using a field emission SEM. Fracture surface of samples was selected from the optimized condition of the experimental for evaluated porosity, grain size, grain boundary diffusion and surface diffusion. The sintered samples were coated with a thin layer of Au previous to imaging.

9) The sintered samples were ground into a fine powder using a ceramic mortar and pestle and analyzed using FTIR technique. The sample selected from the optimized condition of the experimental using powders approximately 0.8 g for chemical analysis. An appearance of chemical groups of sintered samples was investigated in the spectra region 4000 to 400 cm^{-1}.

10) Chemical analysis to determine the Ca/P mole ratios in sintered samples using XRF technique. The powders were used about 5 g for each condition.

2.2.5 Preparation of laboratory rats

This study was approved from Ethical Committee by Faculty of Medicine, Chiang Mai University and we have followed to the principles of the 3Rs by study in laboratory rats. Thirty-two healthy adult (1 to 2 months of ages) non specific genders of Wistar rats, weighing about 300 to 400 g, were used in this study. Laboratory animals of this research were obtained from Laboratory Animal House, Faculty of Medicine, Chiang Mai University. The animals were followed with the instruction of the guide for the care and use of laboratory animals. Each rat was treated in the animal room. Environmental conditions in the animal room were temperature 21 ± 1 °C, and relative humidity of 50 ± 10 %. The light intensity in the animal rooms gave about 325 lux at 1 meter above floor with a light: dark cycle was 12:12 and the fresh air changes per hour in animal rooms was 10 to 15 ACH and it has been guided that noise levels be retained below 85 dB. After 1 weeks of accessed to food and water without restriction before testing [77].

2.2.6 Preparation of samples for implantation [78]

1) The disc samples were cut and lathed into cylindrical shape and may range from 1 mm to 6 mm in diameter and from 7 mm to 10 mm long. These shapes were depending upon the relative size of the species under study (**Figure 9**).

Figure 9 Samples for implantation

2) The samples were cleaned for remove all surface contaminations with appropriate solvents and wash all samples were washed in distilled water before use gamma sterilization.

3) After final preparation and sterilization, handle the test and control implants with great care to ensure that they are not scratched, damage or contaminated in any way prior to insertion.

2.2.7 Processing of implantation and sample collecting

1) The samples were inserted into subcutaneous tunnel of the back of neck of each Wistar rat by making a small incision approximately 1 cm long in skin on either side of the back of the rat's neck respectively. The samples used in the experiment must be those with the optimized condition of each condition. The surgery was done as follows. In each rat, two samples were implanted separately at the right side and the left side of subcutaneous tunnel. One was an optimized sample of one candidate; the other was an optimized sample of another candidate. At least four rats with those two implanted samples were required for each implantation period. The implantation periods were 3, 7, 14, 21, 30, 45, 90 and 180 days (**Figure 10**) [65, 78, 79].

2) Surgery was processed under Tiletamine 50 mg/1 c.c. mixed Zolazepam 50 mg/1 c.c. dose 40 mg/kg and Xylazine HCl dose 5 mg/kg. The dorsal of neck was shaved with area about 1x1 square inch, washed and disinfected with 70 % of alcohol and povidone iodine solution. Then, the wound was closed carefully with absorbable suture to prevent moving of the samples. Waiting laboratory rats were recovery anesthesia. Simultaneously, penicillin G and streptomycin were used to prevent bacterial contamination with doses of antibiotics 20,000 IU/kg after operative. Finally, keep the individually marked laboratory rats in standard cages (266 × 425 × 185 mm^3 of size and 820 cm^2 of area) [77].

3) After a euthanasia process, at necropsy record any gross abnormalities of color or consistency observed in the tissue surrounding of the samples. Remove each sample which was wrapped with surrounding tissue and including the tissue samples were minimum of 4 mm in thickness layer. The tissue was collected in 10 % formalin solution for fixation of tissues [65, 78, 79].

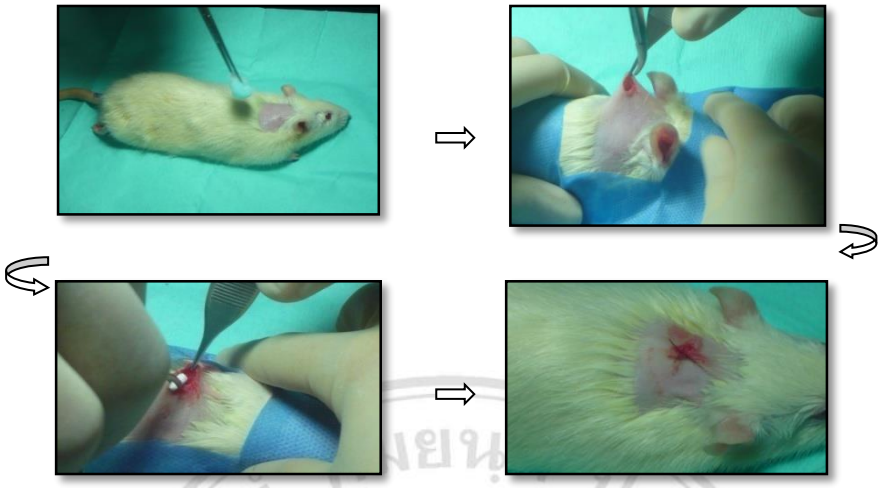

Figure 10 Processing of implantation.

2.2.8 Biological evaluation

1) The tissue was followed standard histology process. The observation for histological examination which included a description of inflammatory cells or cell types and the evaluation of the soft tissue response was done by pathologist. The results from each histological testing shall be evaluated by light microscopic [65].

Pathologist selected use the grading system for foreign body reaction as followed **Table 2.**

Table 2 The histological grading scale for foreign body reaction.

Level of inflammation	Response	Score (symbol)
Mild foreign body reaction	Mild mixed cellular inflammatory infiltration and presence of fibroblasts	1 (♦)
Moderate foreign body reaction	Presence of minimal epithelioid cells with or without small size of monocytoid foreign body giant cells	2 (♦♦)
Severe foreign body reaction	Presence of some epithelioid or large size multinucleated foreign body giant cells	3 (♦♦♦)

The processing of this study was obtained according to the flow chart given by **Figure 11**

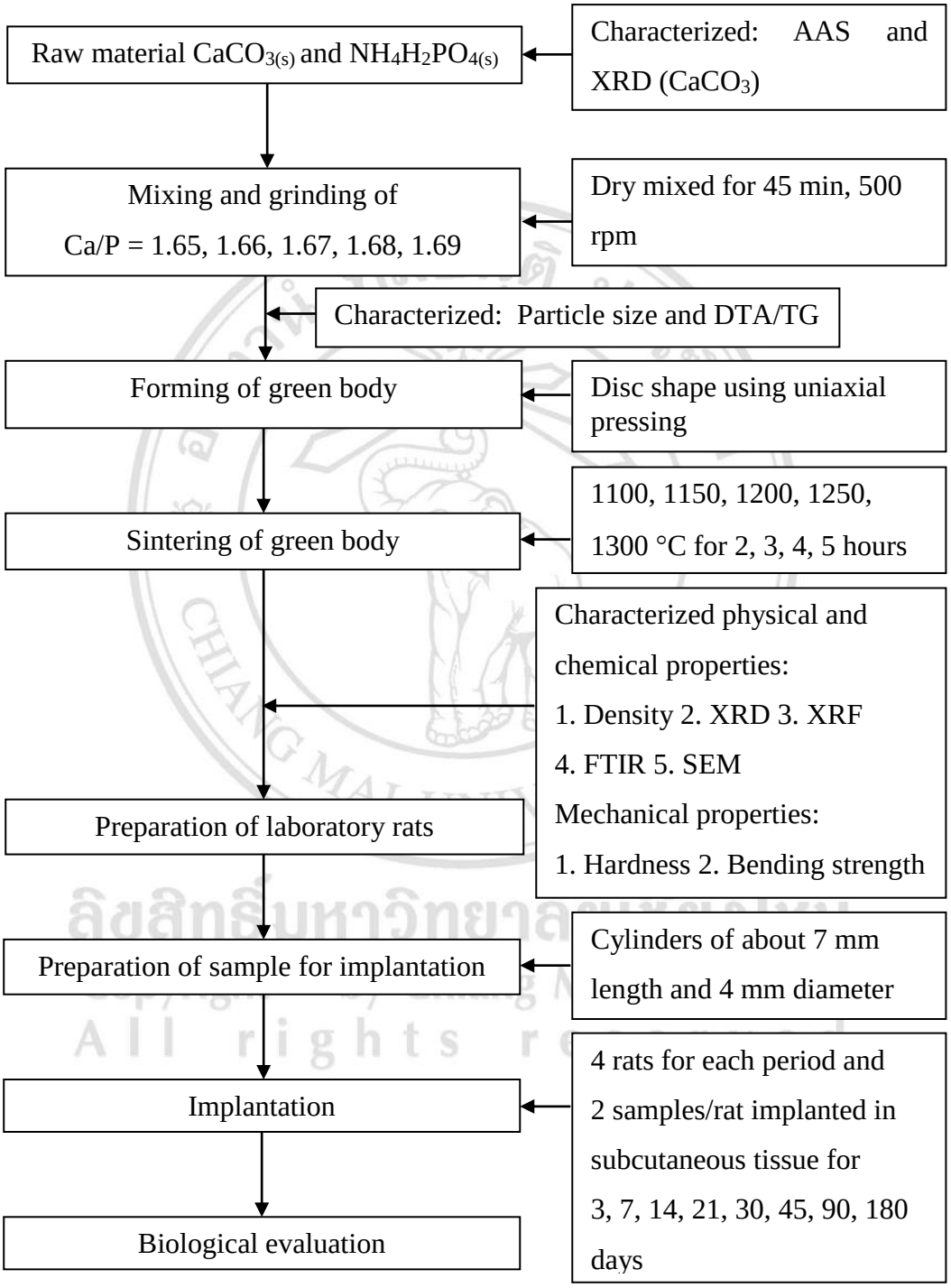

Figure 11 Flow chart of synthetic HA.

2.2.9 Statistical assessment of observed data [80, 81]

Single measurements of the properties of ceramic materials are rarely sufficiently precise to enable decisions to be taken confidently. Tests on a large number of samples are required in many cases. Mean value and variance of samples could be described the population. The equations of variance of samples were defined as follows equation (13) – (15).

$$S^2 = \frac{\sum(Xi-\bar{X})^2}{n-1} \quad (13)$$

where, S^2 = the variance of sample

X_i = data of sample

$\bar{X}$ = mean value

n = number of individual items

Standard deviation is defined as the root mean square value of the individual deviations from the mean.

$$S = \sqrt{\frac{\sum(Xi-\bar{X})^2}{n-1}} \quad (14)$$

$$\text{Standard error} = \frac{S}{\sqrt{n}} \quad (15)$$