EXTRACTION OF EGGSHELL MINERALS FOR BIOMEDICAL APPLICATIONS

BY

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ABSTRACT

Bone diseases are one type of the defects of the body that cause bone weakness and fracture. The medical approaches for bone diseases could be surgical and non-surgical. In severe cases, the patients are treated with surgical approach, which could involve bone transplantation using the autograft, which is taken from the patients' body, the allograft, which is harvested from the other donor or cadaver and transplanted to the patient, and the xenograft, which is a bone graft that is taken from the donor of other species. However, the aforementioned bone grafts still possess several drawbacks such as limited supply, disease transmission, and immune rejection. Therefore, this project is aiming to study the natural hydroxyapatite (HA) materials that could be used as xenografts for bone transplantation. In this research, hen eggshells were collected from KMITL canteen and washed with tap water. The membrane of the eggshells was removed manually prior to the treatment with sodium hypochlorite (NaOCl) to remove the organic component. Thermogravimetric analysis was carried out to characterize the inorganic content and the decomposition temperature of the processed eggshells. The NaOCl-treated samples were heat-treated at 900°C for 2 hours, after which calcium oxide (CaO) was obtained. CaO was dissolved in deionized water and reacted with phosphoric acid (H_3PO_4) at the molar ratio of 1.67. The mixture was allowed to precipitate overnight before being filtered and dried. FTIR spectra have shown that the final samples were plausibly the hydroxyapatite (HA), which was as expected. The final part of this study was to make the HA/polymer composite films on the biomedical 316L stainless steel using dip coating method, which could be another application of HA from this study in addition to the xenografts.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Terms
NaOCl	Sodium hypochlorite
$CaCO_3$	Calcium carbonate
CaO	Calcium oxide
OH^-	Hydroxyl group
PO_4^{3-}	Phosphoric acid
HA	Hydroxyapatite
TGA	Thermogravimetric analysis
FTIR	Fourier-transform infrared spectroscopy
PEO	Polyethylene oxide

CHAPTER 1

INTRODUCTION

This chapter begins with the introduction of clinical problems, which are the major motivation of this project. Subsequently, the objectives of this study are described followed by the scope of this project.

1.1 Statement of the problems

Bone diseases are associated with the defects of the system inside the body and the destroyed skeleton, which results in bone weakness until they fracture. Bone diseases are mostly found in a group of people who are overweight, people who have an accident that directly affects the bones, and elderly groups [1] who begin to have an abnormal function in the body system, and their bones gradually lose density [2]. Common bone diseases in adults and children include the following:

Herniated Disc caused by degeneration of the vertebrae joints, the weakening of the ligaments and a single excessive strain or injury. If this degeneration occurs, the disc could be ruptured, even if there is only minor strain. Moreover, there will also be an increase in the thickness of the surrounding membrane tissue, the reduction of herniation lengths, as well as the rupturing of the tissue causing the herniated disc to slip out of the original position, resulted in severe pain from the compression on the spinal nerves [3].

Osteoporosis is an asymptomatic condition and mostly occurs in women rather than in men. It often damages the bone with unconsciousness until the bone becomes fractured, or occurs unnatural curvature of the vertebrae due to bone loss leading to weak bones that are more likely to break and shorten [4]. This currently affects more in women than men because of the loss of estrogen in female hormones after premenopausal around 25% of women older than 60 years including people undergoing ovarian surgery before the age of 45. When individuals reach the age of around 50, the tissue and calcium of the bone will

decrease 1-3% every year then making it a less dense and increased risk of fracture. Furthermore, there are still other risk factors that can cause osteoporosis such as genetic, gender, age, vitamin D deficiency, often drinking alcohol and caffeinated beverages, smoking, dieting [5], using steroids to treat Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis, taking medicine for a long time, and even children may be at risk of developing juvenile osteoporosis [2].

Spondylolisthesis, this disease causes one of the lower vertebrae to move forward onto the bone directly below it. In some cases, may press on the nerve causes back pain, one or both legs are weak (anesthesia). The risk factors that can cause Spondylolisthesis such as heredity, age, lifestyle, congenital disorder, rapid growth during adolescence, accidents on the vertebrae, degeneration, spinal fracture of a bone structure called the pars interarticularis, infections that spread to the vertebrae, and playing sports such as football, gymnastics, weightlifting, track, and field may also cause stress on the lower back [6].

Rickets is the softening and weakening of bones in children, associated with abnormal growth of bones. The risk factor that can cause rickets is that the body doesn't get enough calcium, phosphorus, and vitamin D or has problems using minerals properly at the growth plate make children have broken bones, twisted, failure to grow, abnormally curved spine, bone deformities, and damage to several organ systems. Premature infants are at risk for rickets, due to getting not enough calcium and phosphorus early to birth because they have less time to receive the vitamin from their mothers in the womb [7].

The main cause of bone diseases is associated with abnormalities of the bones and joints caused by injuries to the joints and bones, infection, vitamin D deficiency, degeneration, and genetic abnormalities [1].

According to the worldwide statistics of patients associated with bone disease, the rate is increasing every year and occurs in female's menopause more than males, as shown in Table 1 below [8].

Table 1: The worldwide statistics of patients associated with Osteoporosis by continents, according to [8].

Continents	Statistics
Europe	The number of QALYs (Quality Adjusted Life Years) lost annually due to fractures in the EU will increase from 1.2 million in 2010 to 1.4 million in 2025, an increase to 20%.
North America	Approximately 80% of these people are women. And rise to 12 million individuals by 2010 and to increase by 14 million by 2020.
Latin America	From 1990 to approximately 2050 the number of fractures for women and men aged 50-64 years in Latin America will increase by 40% and the group older than 65 years will increase to 70%.
The Middle East and Africa	Despite wide sunshine, the Middle East and Africa register the highest rates of rickets worldwide approximately 25-30% in western populations.
Asia	Approximately 50% of all osteoporotic hip fractures will occur in Asia by the year 2050 because the adult Asian intake calcium is less than 1,150 mg/day.
Oceania	Approximately 51% of women aged 60 years or more will increase the number of osteoporotic fractures by over 30% between 2007 and 2020.

World Health Organization (WHO) estimates that in 2000 around the world there will be 1,700 osteoporosis patients and 2,693 per 100,000 population that found in females more than males. World Health Organization's Scientific Group on Rheumatic Disease found that patients with this disease are aged over 45 years and 50% are older than 65 years

[9] and have a mortality rate of around 20-25%. While more than 1 in 3 patients is unable to return to normal life and about 1 in 5 are bedridden patients. The number of bone diseases in the world will increase to 6.25 million and in Asia approximately 3.25 million in 2050 [10]. In Thailand, the statistics of patients with the bone disease have increased in 2010-2012 from the Ministry of Public Health, as shown in Table 2 below [11].

Table 2: Statistics of Thai people associated with a bone disease in 2010-2012, according to [11].

Year	Population in Thailand	Number of patients
2010	57,999,708	3,960,543
2011	58,492,882	5,046,774
2012	58,592,163	7,266,284

From the above information, it is thought that there is an importance of bone disease throughout the whole world and in Thailand. Since the number of patients is significantly increasing every year, and most of the bone substitute materials are imported from abroad which caused a higher price in bone operation cost [11]. The project was conducted to develop the bone materials that have been used in bone operation by extracting the bone minerals called hydroxyapatite (HA) from the wasted eggshell. This product is also believed to be effective in reducing the bone operation cost as well.

1.2 Objectives of the study

1.2.1 To produce hydroxyapatite (HA) from calcium oxide (CaO) derived from eggshell.

1.2.2 To produce hydroxyapatite (HA) from calcium carbonate ($CaCO_3$) derived from eggshell.

1.2.3. To produce hydroxyapatite/polymer composite for biomedical applications.

1.3 Scope of the study

1.3.1 Raw materials used in this study are hen eggs obtained from KMITL canteen.

1.3.2 The raw materials are processed using chemical and thermal methods.

1.3.3 Samples are characterized using Fourier-transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA).

1.3.4 The processed samples are subsequently used to produce organic/inorganic composite films dip-coated on biomedical 316L stainless steel.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to bones

Bone can be categorized into 2 different types which are axial bones and appendicular bones. The axial bones are found in the head and the body that make up the spine, rib cage, and skull, while the appendicular bones are making up the limbs, those being the arms and legs as well as pelvis and shoulder [12]. Another way to classify the bone is by looking at its shape, as it can be long, short, flat, irregular, and sesamoid bones as shown in Fig.1 [12]. Long bones are longer in length rather than wide, like the limbs, while short bones are cube-like and can be found in the ankles and wrists. Flat bones are thin and curved which can be found in sternum and shoulder blades. The irregular bones are having complicated and unpredictable shapes such as vertebrae and hip bones. The sesamoid bone is having the shape as it was named, its shape is small and round as the sesame. This kind of bone can be found in tendons as it helps overcome the compression forces. The main purpose of the bone is to provide the support the body structurally and protect the inner organ such as the heart that has been covered by the rib cage. Besides, bones also act as a lever to allow the performing in the physical tasks. Bones also provide the suitable environment for the bone marrow in which the blood cells are created and serve as a mineral storage, like calcium and phosphate, which can be released into the bloodstream [12, 13]. Moreover, it acts as a fat storage, hormone production, and blood cells formation as well. Bone is one kind of hard tissue, but what makes bone different from other kinds of hard tissue are collagen and calcium phosphate which are the principle components of the bone [13].

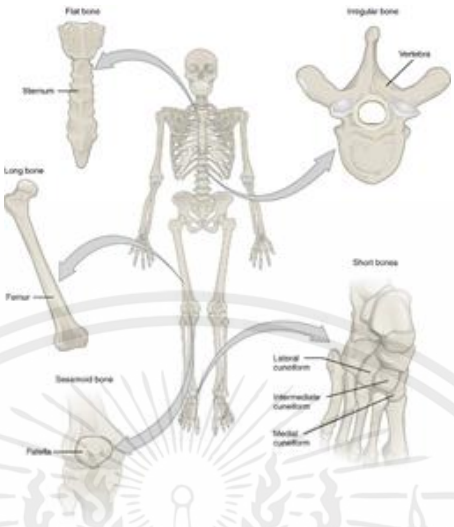

Figure 1: Classification of bones [12]

Bone is an organ because it is made up of several types of tissue. Most of the bone is made of bone cells or osseous tissues, but it also contains the nervous tissue, connective tissue, cartilage, and blood vessels. The outer layer of any bone is made of compact bones, which is smooth and dense, while the inner layer of the bone is made of spongy bone, which is like the pore of honeycomb with lots of needles inside [12]. The combination of compact bone and spongy bone provide strength and compression in response to stress, respectively. Typically, the open spaces within the bone will be filled with bone marrow. Each type of bones consisted of the different arrangement of compact and spongy bones. The short, flat, and irregular bones are covered by the compact bone with the spongy bone lying underneath in the form of thin plates [14]. There is no cavity for the bone marrow to be located, and hyaline cartilage covers portions of the surface that are involved with joints. Long bones are different from the rest as they contain a tubular shaft, called a diaphysis, which is located between the proximal and distal ends of the bone [14]. It is made up of a thick collar of compact bone surrounding a medullary cavity, or marrow cavity. In adults, this cavity contains yellow bone marrow which is richly in fat [12, 14]. Both ends of the long bone are called the epiphysis which contain spongy bone inside the compact bone, and cartilage covers the joint surface for cushion and stress absorption. Apart from the yellow marrow bones, there are red marrow which can be found within the cavity of the

spongy bone, and this type of marrow also produces the blood cells [12]. Another region of the long bone is metaphysis, it is located between the diaphysis and each epiphysis. This region represents the narrow area that contains the epiphyseal plate, a disk of cartilage that grows during childhood, which is longer as a child gets taller, that is why the epiphyseal plate is also being called the growth plate [12, 14]. When the bones stop growing in adulthood, the cartilage that covers the epiphysis will be replaced by the osseous tissue and the epiphyseal plate will become epiphyseal line [12]. The outer layer of the bone was covered with a white membrane called the periosteum. It consisted of the fibrous layer made of dense irregular connective tissue, and an inner osteogenic layer containing the primitive stem cells. The periosteum is attached to a network of nerve fibers, blood vessels, and lymphatic vessels, which then pass through the diaphysis to the marrow cavity, and perforating fibers connect the periosteum to the bone [14]. The endosteum which lies in the medullary cavity is a location where the bone growth, repair, and bone remodeling occur. It covers the internal spongy bone layer, as well as the canals that pass through the compact bone [14].

To protect the internal organ, the flat bones are organized in three layers as shown in Fig. 2 which have the compact bone on the top and bottom while having the spongy bone in between. This is effective when there is a fracture on the outer layer of the bone, the internal organ still be safe by the protection of the rest of the bone [12].

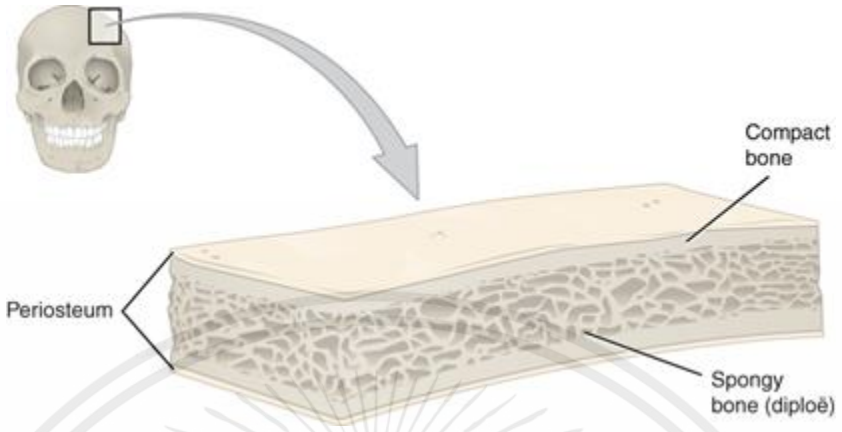

Figure 2: The cross section of flat bone [12]

As mentioned above, bones consisted of compact bone tissue in the outer layer and spongy bone tissue in the inner layer. Compact bone tissue consists of units called osteons or Haversian systems, which are formed in cylindrical shape and aligned in parallel to the diaphysis of the long bone [12]. It contains mineral matrix and living osteocytes which function in transporting the blood. Each osteon consists of concentric rings of calcified matrix called lamellae that surround a central canal or Haversian canal, as shown in Fig. 3a and Fig. 3b, which contains blood vessels, nerves, and lymphatic vessels. These vessels and nerves lie perpendicular to the perforating canal thus it helps to extend to the periosteum and endosteum [14]. The alignment of osteons in compact bones help the bone to resist bending and fracturing. The compact bone also serves as the calcium storage which could absorb, and release calcium as required [12, 14].

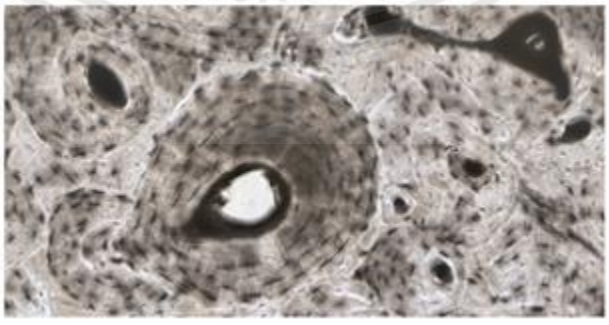

Figure 3: Components of compact bone tissue [14]

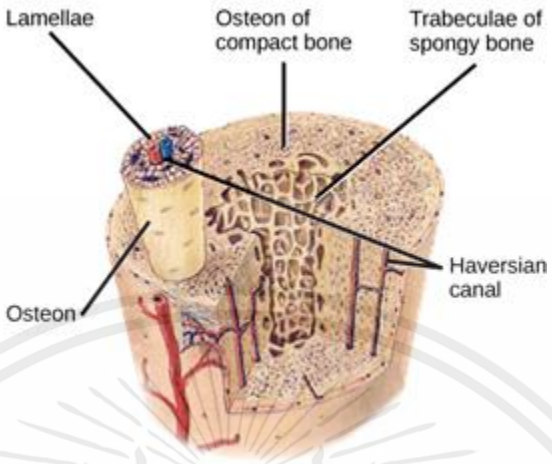

Figure 4: The Micrograph of Concentric Lamellae and Central Canal. LM × 40. (Micrograph provided by the Regents of University of Michigan Medical School © 2012) [12]

Apart from the compact bone tissue, there is spongy bone tissue that consists of trabeculae which are the place where osteocytes and lacunae can be found. It is a lattice-like network of matrix spikes which is not aligned in concentric circles [12]. The red bone marrow is found between trabeculae. This spongy tissue contains blood vessels which are responsible for delivering nutrients to the osteocytes and removing the waste. Spongy bones are light in weight and low density, thus will help reduce the whole bone density [14]. The reason why the spongy bones are light in weight is because there are spaces of the trabeculated network within the structure and this will provide the balance to dense and compact bone. As a result, the movements occur easily as well as allow the epiphyses of a long bone to compress when the stresses are applied. In addition, spongy bone is an area where the stresses arrive from many directions [12].

Microscopic anatomy of the bone shows that the bone consisted of 4 types of cell: osteogenic cells, osteocyte, osteoblasts, and osteoclast [12]. Each type of cell serves a unique function and occurs at the different locations of the bone. The osteogenic cell is one type of stem cells that are actively divided, and they are located at the periosteum and endosteum. If the bone is growing, they will become flattened, and this type of cells can

differentiate into other types of bone cells such as osteoblasts. The osteogenic cells are undifferentiated with high mitotic activity which means that they are the only type of bone cells that divide. The next one is osteoblasts; it can be found in the periosteum and endosteum which represents the growing portion of the bone [12]. It is responsible for bone growth, but it does not divide into other types of bone cells. This type of cell can synthesize as well as secrete the matrix containing collagens and other proteins which play the important role in bone growth. Osteoblasts are trapped within the calcified secreting matrix and transform into osteocytes by changing its structure [12]. Moreover, it represents as an actively mitotic and cube-shaped while active. Unlike the osteoblast, the osteocytes lack of mitotic activity. It is a primary cell of the mature bone and the most common type of bone cell that is located in lacuna. This serves a function in maintaining the mineral concentration of the secreted matrix, while they are capable of communicating between other cells and receiving the nutrients from the cytoplasmic processes as well. While the new bone cells are formed, the old and injured cells are dissolved in order to repair or release the calcium [12]. The osteoclast is a type of cell that can be found on the bone surface. It is responsible for bone resorption or breakdown processes. Instead of originating from the osteogenic cells, the osteoclasts originate from the monocytes and macrophages. As the information above tells, the bone cells are generated by the osteoblast, while the osteoclasts are keeping damage to the old bone. This ongoing process between two types of bone cells is responsible for reshaping the bone [12].

2.2 Bone disease and current treatment

A bone fracture is often treated with a splint. A Splint will be used as an external body support and locks the unnecessary movement. Furthermore, a splint is used to encourage healing by placing the injured limb in the right position, to reduce swelling, and to encourage the bones to align (straighten) [10, 2]. In some cases, cannot use a splint such as a small bone (toes or fingers. So, using medication to reduce the pain of the fracture is the suitable way to cure the patient [11].

Nowadays, bone disease can be prevented in many ways to reduce the causes of complications in the future [2, 11].

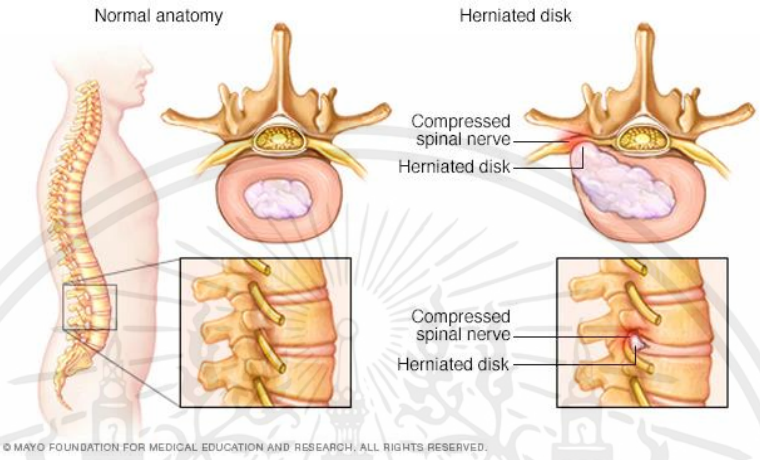

Figure 5: Herniated disc [15]

Herniated Disc caused by a disc located between the individual bones (vertebrae) is destroyed until press the nerve. When a herniated disc pressed on the nerve, it can result in pain, weakness, and Numb in an arm or leg, Which discs are circular like small pillows with a strong, outer layer (annulus) that surrounds the nucleus and located between vertebrae, discs act as shock protection [15]. The risk to make herniated disc is excessive body weight, carrying heavy loads, genetic disease, being hurt or force that affects a back injury, and loss of flexibility by smoking [15, 16].

Herniated Disc treatment will improve the bone of the patients in 2-3 months depending on the severity of the symptoms and the position of the herniation, which can be classified as follows [15, 16]:

1. Pharmacologic Therapy using painkillers to treat patients with mild to moderate back pain can be obtained by taking ibuprofen or naproxen. If the symptoms do not subside, the doctor will need to prescribe narcotics to reduce the pain such as Codeine or Paracetamol, which contain oxycodone [16]. If the pain in the legs, hips, or buttocks which is located along the sciatic nerve, the doctor will need to prescribe high-altitude medication

tablets, for instance, Antidepressant and Anticonvulsant to reduce nerve pain associated with a herniated disc [17]. However, both medicines cannot be used to treat all the patients as some medications may cause side effects to patients [15, 16].

2. Physical therapists help patients move better and prevent back injuries by using techniques for improving functional movement and pain relief such as massage, bending the joints, and stretching exercises. Pain medication and muscle relaxants may also be beneficial in conjunction with physical therapy [16]. If non-surgical treatment options, such as physical therapy and medications, are unable to reduce the pain. So, the surgical options are the best way to allow the patient to return to normal life again [17].

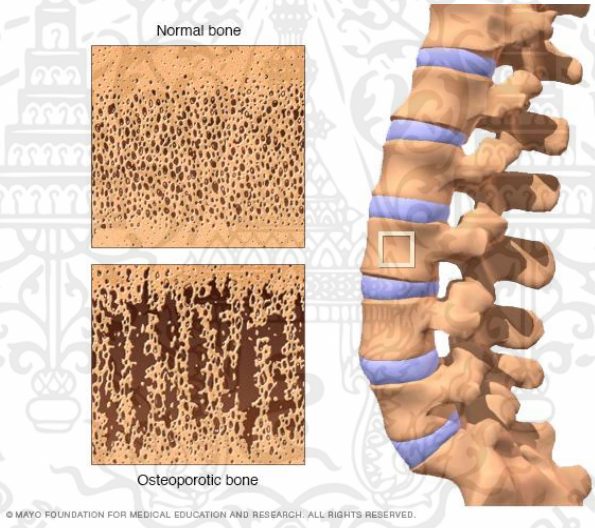

Figure 6: Osteoporosis [18]

Osteoporosis is changing in bone structure, a condition in which bone mass decreases, also increases the risk of fractures associated with aging, and gonadal hormone deficiency, such as menopause, estrogen deficiency, due to bones act structure of the body and main reservoir of calcium [18]. In childhood and adolescents with a higher bone formation rate than the bone resorption rate, the bone mass continues to increase until the peak bone mass at the age of 20-30 [18]. After that bone formation rate is equal to bone resorption rate processes also bone mass remains constant until about 40 years of age the

rate of bone resorption will more than bone remodeling then resulting in decreased bone mass [19].

Osteoporosis Treatments to reduce the risk of osteoporotic fractures include [18]:

1. Lifestyle Measures are the main way to reduce bone loss, reduce fracture risk, and improve bone mineral density. In adults, particularly among postmenopausal women need at least 1,200 mg of calcium per day and 800 IU of vitamin D per day. Eating a balanced diet is important for health from each of the five food groups and will get a mix of the best nutrients and vitamins together with exercising at least 30 min per day to reduce the risk of bone fractures [18, 20]. Furthermore, the cessation of smoking is the main factor in reducing bone loss and normalizing estrogen levels in the blood [18].

2. Pharmacologic Therapy can inhibit osteoporosis. The first group is Bisphosphonates to inhibit bone resorption and has few adverse effects. The study found that Alendronate, Risedronate, and Zoledronic acid be able to increase bone mineral density and can be used to reduce the risk of hip and spinal compression fractures in osteoporosis [19]. The second group is Parathyroid hormone/parathyroid hormone-related protein analog (PTH/PTHrP) that medicine to stimulate new bone formation (anabolic agent), reduces the risk of fractures and increases bone mineral density more than other drugs. The PTH and PTHrP hormones increase dietary calcium absorption and, in the kidneys, increase bone resorption to store calcium levels in the blood. Furthermore, the PTH hormone stimulates 1,25-dihydroxy vitamin D, which increases the absorption of calcium in the intestines [20]. The third group is Denosumab, a human monoclonal antibody to receptor activator of nuclear factor kappa-B ligand (RANKL) or blocking a protein. Which receptor of osteoclast that will break down old bone material and inhibit osteoclast resulting in reduced bone resorption, improve bone mineral density, and reduce bone fractures. The last group is Selective estrogen receptor modulators (SERMs) that can bind to the estrogen receptor and stimulate each organ such as Raloxifene, Bazedoxifene, and Tamoxifen [20]. Especially, Raloxifene is most commonly used if it is used continuously for 8 years will be safe. The estrogenic effect at the bones can reduce bone

resorption, improve bone mineral density, and reduce the risk of spinal fractures but cannot reduce fractures in other positions [20]. Therefore, SERMs are one way for an optional drug to be used more efficiently if patients cannot use bisphosphonate [20].

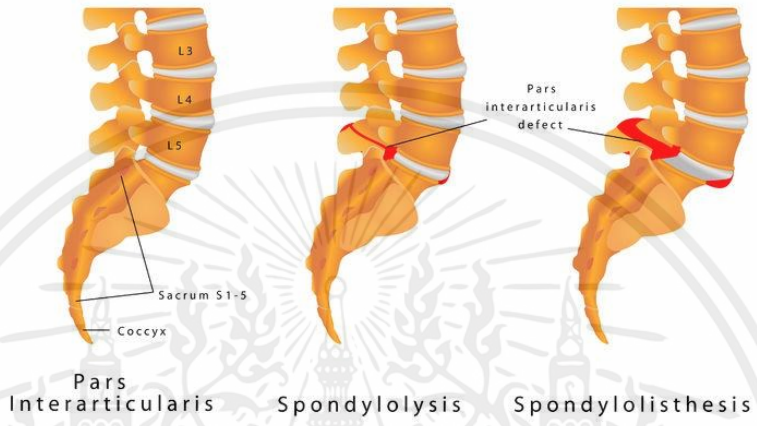

Figure 7: Spondylolisthesis [21]

Spondylolisthesis is a condition where one vertebra is more forward than normal (maybe moving back but very rare), often found in patients aged 50 years and more often in females than males, due to females have less joint stability, muscle, and tendon strength than males. So, females are easier to suffer from this disease [21]. The fourth and fifth lumbar spine dislocation (L4-L5) is most commonly found because this spine is heavily loaded, higher stressed and more active than other joints. In some case, nerve compression resulting in pain back, numbness, weakness in one or both legs, which depending on lifestyle, congenital disability, accident, degeneration with age, rapid adolescent growth, stress activities that accumulate in the lower back, especially sports such as football, gymnastics, athletics, and weightlifting. Including Infections such as spinal tuberculosis or cancer that spread to the spine [21].

Spondylolisthesis Treatment depends on the severe symptom, which can be classified as follows:

1.Pharmacologic Therapy uses ibuprofen (Motrin®) or naproxen (Aleve® or Naprosyn®), which has been recommended in helping to reduce pain and inflammation (irritation and swelling) if the patient has numbed in the leg, corticosteroids may inject at the nerves that are pressed by the spine [22].

2. Physical Therapy focuses on reducing pain and exercising the back muscles to strengthen muscles, support the spine and be able to return a better position. Especially core stabilization training is a deep muscle strength training that attached the spine to be strong. Furthermore, using accessories such as back support and Kinesio tape on the back to support the back muscles and remedies to relieve back pain [23]. If non-surgical treatment options, such as physical therapy and pharmacologic therapy are unable to reduce the pain, so surgical options are the best way to allow the patient to return to normal life and reduce the risk of the bones by nerve decompression surgery and connect the spine or insert the metal rods at the spine. The goals of surgery for spondylolisthesis are to reduce the pain associated with a nerve, to stabilize the spine, and to increase the person's ability to function [22].

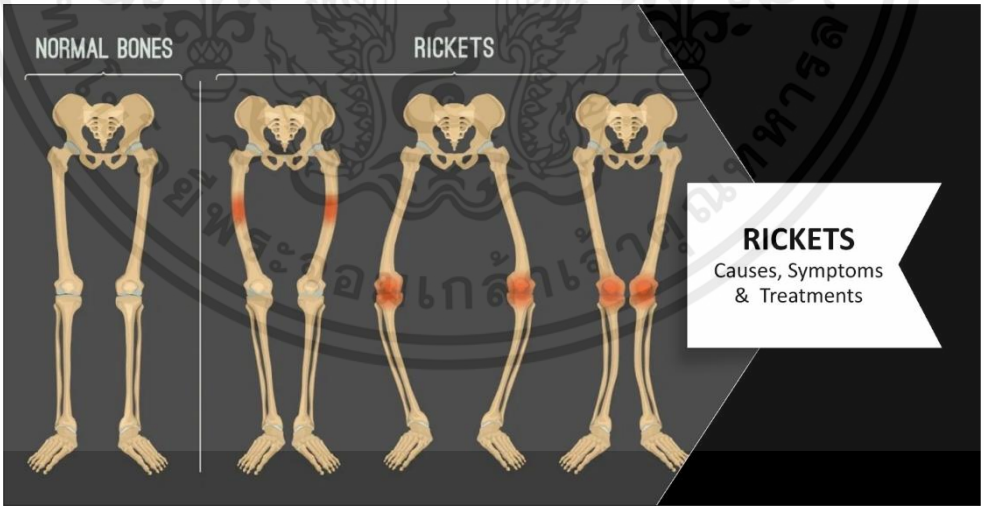

Figure 8: Rickets [24]

Rickets is a condition in which cartilaginous and curvature of the bones by abnormalities in the production and breakdown of vitamin D, prosperous, and calcium in the bone before the closure of the growth plate, causing the bones to become weak and decrease absorption of calcium and phosphorus, which of these minerals are a component of bone. These causes soft and fragile bone tissue to prevent growth, resulting in deformed, distorted, and broken bones [25].

Rickets is most common in children aged 6-36 months because the body is developing bones, muscles, and organs, So patients need continued and appropriate treatment to prevent chronic or permanent bone damage where the most affected part is the epiphysis. In adults, this condition is called osteocalcin. The most common causes are vitamin D deficiency, hypophosphatasia, possibly severe diarrhea, and vomiting, including some kidney diseases [25].

Rickets treatment focuses on vitamins and minerals such as fish, offal, dairy products, eggs, supplements, and should be exposed to sunlight or outdoors to synthesize vitamin D from sunlight by providing 400 IU of vitamin D per day. In premature babies, 200 IU of vitamin D if abnormal leg bones (bowed legs) should orthopedic splint before medication. In the case of premature babies, who are unable to take medicine or have problems with intestines and liver, vitamin D must be injected once a year depending on age and the cause of the symptoms [7, 25].

In the case of premature babies, who are unable to take medicine or have problems with intestines and liver, vitamin D must be injected once a year depending on age and the cause of the symptoms. However, if there are severe abnormal bones on the leg, the surgical option will be performed to align the bones in the right position [25].

Current methods of treating bone diseases compose of a surgical and non-surgical option such as physical therapy, medications, and wearing a cast to fix the position of bones, joints, and other related structures. Furthermore, repair broken bones, and rearrange the bone to the right positions to prevent movement, reduce pain, swelling and protect the

bone from other hazards. Non-surgical treatment options, such as physical therapy and medications, are unable to reduce the pain because some surgical may affect other parts of the body. So, the surgical option is the last choice to treat bone diseases [7, 25].

2.3 Critical size of bone defects

Bone defects are the abnormal conditions of bone tissue, which often occur after experiencing high grade open fractures with bone loss, extensive trauma, blast injuries, and subsequent infection requiring debridement of bone. Bone defects are considered to be a serious illness as the vital components of the bone are damaged by the pathological process. [26]. This will continue to be a significant burden of disease management associated with the bone defects, specifically if the bone defects are critical sized [26, 27]. The clinical treatment of bone defects is controversy in terms of the size of the defects and how to determine whether the defects are considered to be critical sized. There is still no standard definition of critical-sized defect. In general, the "critically-sized" defect can be defined as "*one that could heal spontaneously despite surgical stabilization and requires further surgical intervention*" [27]Some of the suggested definition has been stated that it is the smallest size of the bone defect that will show the sign of regeneration less than 10% throughout the lifetime [27]. There is a biological action that look quite similar to critical-sized defect, but it does differ in somehow, which is called nonunion. Nonunion region is *"an impaired region where cellular and molecular signaling and biomechanical instability that often occur without a bone gap"*, whereas the critical-sized defect is referred to *"an adequate biology but an inability to replace substantial bone loss that may be complicated by the soft-tissue environment and patient demographics"* [28]. Another differences between those two is the requirement to manage the defect. In other words, the critical-sized bone will always require defect management, whereas the management is not always required in nonunion cases [28]. Critical-sized defects can develop into atrophic nonunion due to its nature of the fracture, while an atrophic nonunion may initially occur without any bone loss. There are numeral factors that can affect the critical-sized bone defects,

including the circumferential loss of bone, anatomical location, the soft tissue environment and surrounding muscles, age, the presence of chronic diseases, and other comorbidities [29]. The anatomic location between femur and the tibia will also affect the effectiveness of the clinical outcome. The spontaneous healing of femoral segmental defects could be observed with 6 – 15 cm long due to the good soft tissue environment [30]. In contrast, poor clinical outcome is usually observed from the tibia due to the lack of spontaneous healing and smaller critical size, which is around 1 – 2 cm or 50% of the cortical circumference. This could be a result of poor soft tissue coverage and blood supply in tibia. It is necessary to consider the defect size which can be illustrated by the radiographic apparent bone gap (RABG) in order to assess whether or not defect management is required. The RABG was determined by the average bone gap on 4 cortices. A RABG of 25 mm was the optimal threshold for separating between the outcomes of union and nonunion. The reports claimed that the fractures with a RABG of less than 25 mm achieved union much more frequently than those with gaps greater or equal to 25 mm [31].

2.4 Bone Transplantation

Bone transplantation sometimes refers to bone grafting. It is a surgical procedure aiming to fix the bones that are damaged from the trauma [32]. The process was done by transplanting the bone or bone-like materials from one area to another area in purpose of healing, fixing the old bone, growing the new bone around the implanted device, filling the area where bones are missing, and providing structural stability. Bone grafting is necessary when the natural healing process of the bone is not working for each injury site. In other words, the fractures are too large, so that the natural healing process is not effective anymore [33]. Bone or bone-like materials performed in bone grafts may come from the patient itself, from a donor, or it can be entirely synthetic. Since the demand of bone replacement materials or bone-like materials is increasing every year, the research proves that the calcium phosphate-based materials especially hydroxyapatite (HA) which have the

similar components as the natural bone are suitable in producing the bone-like materials [33].

There are three types of bone graft: autograft, allograft, and xenograft which have a different approach in harvesting the bone. Autogenous bone graft, or sometimes being called autograft, which is a surgical procedure where the bone is taken from the patients from one location and transferred to the area of injury. The graft bone is mainly harvested from the iliac crest, and other sources such as the proximal tibia, fibula, or rib can be used as well [34, 35]. It is the most preferable surgical procedure in most cases as it provides the low risk of disease transmission. The reason is that this process provides calcium scaffolding for the bone to grow as well as containing the bone-growing cells and bone-growing proteins to maintain the bone growth in the patient [34]. Autografts maintain the viable osteoblasts and osteoprogenitor cells from the patient's bone and also provide the osteoconductive and osteo-inductive potential. The grafted bone has been supplied with the biocompatible osteoconductive properties of the calcified matrix of mature bone, and organic components such as collagen. The bone morphogenetic protein (BMPs) is one of the bone growth-factors that are primarily responsible for the osteoconductive capacity of autograft. Although this method is considered as a gold standard of surgical achievement [36], there are some drawbacks in this procedure such as it adds another surgical site which will lead to the additive pain and discomfort. The surgical wound is also included in some of the drawbacks as it might be caused by infection or disease transmission through the surgical wound. In some cases, the patient might have a problem with nerve injury and bleeding, but it is rarely found. The important thing in autograft is that there is limited bone supply and sometimes it needs to be supplemented with some form of bone graft substitute [28]. But importantly, autograft bone itself does not pose a risk of disease transmission through the transplanted bone and its procedures show a high rate of success for certain spinal fusions, such as anterior and posterior cervical arthrodesis [35].

Allograft is a surgical procedure which the bone is harvested from the donor or cadaver and transplanted to the patient. It refers to a medical procedure of transferring the

tissue/bone between genetically nonidentical members of the same species and sometimes the transplants often come from the relatives [37]. To consider the successful rate of bone fusion, allograft is not the best choice to be considered. The reason is that allograft only provides the calcium scaffolding which means that it does not have any bone-growing cells or bone-growing proteins that play the important role in stimulating the new bone growth [38]. The advantages of allograft are lying between an autograft and xenograft; in some cases, the patient's own bone is not enough to supply the bone operation while applying the synthetic bone may not be right for patient because it might provide the properties which is not related to human bones [39]. Unlike the autograft, the allograft does not provide the surgical risks for harvesting the patient's own bone. There are some drawbacks in the process which contain low chance of fusion as it provides only the calcium scaffolding thus the bone-growing cells or bone-growing proteins are absent here in this process. Another drawback is that it provides the risk of disease transmission, there is still a small potential risk of disease transmission from using the donor bone even though the allograft bone is provided by the FDA [38]. Many surgeons are being concerned with the risk of disease transmission via the grafted bone. The concerns exist regarding transmission of human immunodeficiency virus (HIV), hepatitis B (HBV), and hepatitis C (HCV), group A streptococcus, *Clostridium* species, and prions. To prevent the disease transmission, the grafted bone/tissue must be initially sterilized by using Irradiation Technique, applying Ethylene Oxide, or even using the Peracetic Acid-Ethanol. The most common irradiation processes is currently employed by the gamma rays, especially Co^{60}, which induces the chemical reaction such as cross-linking, branching, and grafting, thus will lead to the destruction of pathogens [40]. Another way to approach the irradiation is to use electron-beam instead of gamma rays. Because there are several advantages of electron-beam over the gamma rays such as it has greater control and accuracy of applied dosage, substantially reduces the operating time, and preserves the tissue from the addition of carbon dioxide that releases from the process [40].

Another type of bone grafting is xenograft. It is a bone graft that is taken from the donor from other species such as bovine and is used as a calcified matrix [41, 42]. In recent

years, not only bovine bones can apply to the bone graft but also the porcine bone as well. The advantages of xenograft over allograft are there is a low risk of disease transmission as it has been proved by several research. According to the laboratory research, in vitro tests of bovine cancellous graft showed a high biochemical resistance to axial loads, and osseointegration was observed in an animal model [43]. Numerous studies have been conducted on bovine especially in cuttlebone (CB). It is easy and inexpensive to obtain the surgical procedures, due to its morphology and mineral composition, the CB can be shaped in various formats, it is considered to be compatible with other types of bone structure and it contains high osteo-inductive capacity [44]. Unlike the autograft and allograft, the xenograft does not have the ability to stimulate the new bone cells because it provides only the function of calcium scaffolding. Even though it cannot stimulate the bone growth, but its ability is available in filling the small defects of the bone and the result is that the portion of the graft may turn into the patient bone as the time is passing by [45].

2.5 Eggshell

The egg is a high nutrient of food, complete proteins, and contains essential amino acids. Therefore, popularly used in many types of food, and eggs consist of many kinds such as Hen egg, Duck egg, Quail egg, and fish egg. Most commercially produced is hen's egg, due to easy to find, cheap, and has a high nutritional value [46]. In 2017, Thailand produced more than 16,470 million hen eggs for consumers and food industries [47], which cause abandonment of eggshells and environmental problems such as breeding places for flies and mice also increase ammonia and hydrogen sulfide in the air.

The eggshell is thick 0.24-0.42 mm [48] also, the color of the eggshell depends on the types and breed of poultry. In general, a hen is a yellow egg, a duck is a white egg, and most bird eggshells are spot on eggshells [49].

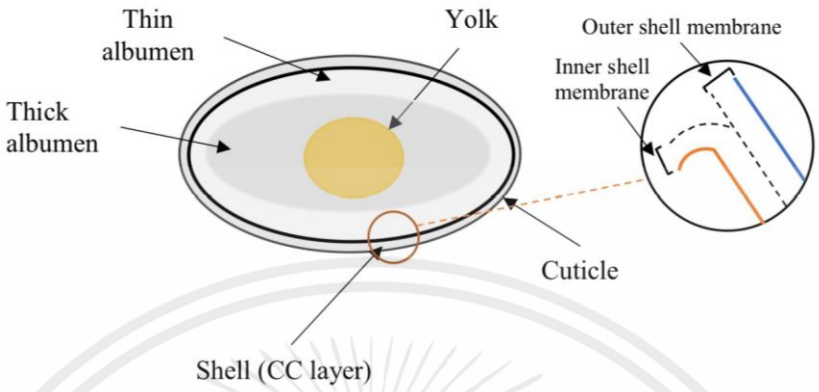

Figure 9: Layer of Eggshell [50]

The eggshell consists of different layers in well-organized position which grows in the various segments of poultry's oviduct. The amount of different soluble and insoluble proteins, minerals, and fibers are deposited during the process of forming an eggshell which will further used in process of developing the embryo. The eggshell composed with three layers: shell, albumen, and yolk as shown in Figure 9 [50]. The inner part is surrounded by the albumen layers which has been covered by the hard eggshell, while the outer layer is composed of a thin film layer, a limestone layer, and two shell membranes (inner and outer membranes). An eggshell composed of 98.2% of calcium carbonate ($CaCO_3$) crystal with some proteins and other minerals [51]. Besides, it also contains some amount of magnesium carbonate ($MgCO_3$) and magnesium phosphate ($Mg_3(PO_4)_2$). The chemical components of eggshell are varied from each type of poultry as shown in Table 3. It has a bumpy and grainy in texture, while it has been covered with as many as 17,000 tiny pores. This eggshell considered to be semipermeable which means that it allows some particle to pass through it, in this case, air and moisture can pass through these pores [51]. The outermost layer of the shell is coated with a thin layer called *bloom* or *cuticle* which help eliminating the external bacteria penetration to a certain extent and prevent the moisture, this layer is about 10 to 20 µm of thickness [50, 51]. The inner and outer membranes are lying between the eggshell and egg yolk, these two transparent layers provide a defense mechanism against the bacterial invasion. Most of the eggshell components are composed

with inorganic limestone substances, 3.3% of protein, and 1.6% of water. The thickness of the inner and outer membrane is 50 μm and 15 μm respectively [50].

Table 3: Chemical components of the different kind of eggshell [51].

Minerals	% (weight)		
	Hen's eggshell	Duck's eggshell	Quail's eggshell
$CaCO_3$	99.0	96.5	97.3
Mg	0.5	0.1	1.0
P	0.2	0.5	1.1
S	0.1	1.2	0.4
K	-	0.0839	-

From the information that has been described above, it was useful to adopt the eggshell to produce the nanoparticles technology that can be widely used in operation especially in bone operation. Million dozen of eggshell have been introduced into the market but surprisingly, there is small amount of eggshell that can be used to produce a beneficial for the human being. The statistic claimed that 17,500 tons of calcium carbonate could be produced from eggshell annually. Consequently, for every 100 million eggs that are discarded, there is ability to produce 650 tons of high-grade calcium carbonate. So, the aiming of this project is to convert the waste materials and turn into the bone substituent materials.

Eggshell can be used as a material to produce a bone substituent material which is hydroxyapatite (HA). While hydroxyapatite itself can be used as a material for bone implantation as well. The application of hydroxyapatite can be done by many approaches

as shown in the Table 4 such as High Velocity Oxygen Fuel (HVOF), electrodeposition, electrochemical, plasma sprayed technique, and dip coating. While dip coating is the best alternative technique that can be applied as one of the applications for bone implantation.

Table 4: Technique for bone implantation application

Article Name	Film - Materials	Methods	Applications
Biological properties of ZnO, SiO_2, and Ag_2O Ternary Dopant Plasma Sprayed Hydroxyapatite Coating for Orthopedic and Dental Applications [52]	HA coated on Ti (Ti6A14V and pure Ti)	Plasma spray	Orthopedic and dental applications.
Fabrication and characterization of nanostructured hydroxyapatite coating on Mg-based alloy by high-velocity oxygen fuel spraying [53]	HA with 250 ml polypropylene coated on Mg-based alloy (AZ61)	HVOF spraying (plasma spray) by initially anodized the Mg substrate	Improve the bioactivity and reduce the hydrogen gas release.
Fabrication of hydroxyapatite/stearic acid composite coating and corrosion behavior of coated magnesium alloy [54]	hydroxyapatite (HA) and stearic acid (SA) coated on magnesium alloy	Electrodeposition and solution method	Improve the protection and enhance corrosion resistance of biomedical magnesium alloys.

HA coating fabricated by electrochemical deposition on modified Ti6Al4V alloy [55]	HA coated on modified Ti6AI4V alloy	Electrochemical methods	Demonstrate the effects of current density on the coating compositions, microstructure, and bonding strength between coating and substrate.
HA coating on Mg alloys for biomedical applications [56]	HA coated on Mg alloys	All of coat technique	Enhancing the corrosion resistance, biocompatibility, and bioactivity of the Mg alloy substrates
Crystalline HA coating on PEEK via chemical deposition [57]	HA coated on Polyether ether ketone (PEEK)	Chemical deposition	Investigate the coating technique and properties for orthopedic applications.
Effect of electrodeposition parameters and substrate on morphology of Si-HA coating [58]	Si-HA coated on Mg-5Zn-0.3Ca alloy	Pulse electrodeposition	The formation of Si-HA was more homogeneous when using Mg alloys with nano-HA as substrate.
Mechanical, biological, and antibacterial characteristics	(Sr,Zn)- HA coated on Ti-	Atmospheric Plasma-sprayed	The coating provides the excellent mechanical and

of plasma sprayed (Sr,Zn) substituted HA coating [59]	alloy (Ti-6Al-4V)		biological performance as well as enhancing the antibacterial properties of the metallic implants.
In vitro characterizations of Si^{4+} and Zn^{2+} doped plasma sprayed HA coatings using osteoblast and osteoclast coculture [60]	HA doped with ZnO and SiO_2 coated on Ti-alloy (Ti6Al4V)	Induction coupled radio frequency plasma spray system	Demonstrates the promising effects of SiO_2 and ZnO on the enhancement of osteoblast proliferation and bone matrix production, as well as reducing osteoclast proliferation.
Characterization of Antimicrobial and Bone Regenerative Activities of Porous Si-nHA Scaffolds Containing Vancomycin and rhBMP2 [61]	HA coat on Si	The wet chemical precipitation reaction	Scaffolds containing Vancomycin and rhBMP 2 enhance new bone formation
Hydroxyapatite/collagen coating on PLGA electrospun fibers for osteogenic differentiation of bone marrow mesenchymal stem cells [62]	PLGA coated on HA/Col (HA/Collagen)	Electrospinning process sprayed onto Ti disc	Improved interaction between mesenchymal stem cells and accelerate their osteogenic activities.

Dip coating assisted polylactic acid deposition on steel surface: Film thickness affected by drag force and gravity [63]	Steel surface coat on HA/PLA	Dip coat method	The dip-coated PLA film thickness decreases with increasing withdrawal speed.
Polyethylene Oxide and Silicon-Substituted Hydroxyapatite Composite: A Biomaterial for Hard Tissue Engineering in Orthopedic and Spine Surgery [64]	Polyethylene oxide (PEO)/silicon-substituted hydroxyapatite (Si-HA) composite	Electrospinning Technique	Develop a scaffold for orthopedic and spine tissue engineering

During the dip coat process, the dip coat solution is needed to be prepared. There are varieties of material that can be used to generate the thin film onto the sample such as Polylactic Acid (PLA), Polylactic-co-Glycolic Acid (PLGA), Collagen, or even Polyethylene Oxide (PEO) [65]. Hence, PEO is considered to be the most suitable materials for the dip coat solution as its compound has been approved by Food-and-Drug-Administration for the medical uses. PEO is a synthetic polymer that a hydrophilic property which means that it was easily dissolved in aqueous solution or inorganic solvents [65]. Its polymer chain can coordinate with the alkali cations such as Li^+, Na^+, Ca^{2+}, etc [65].

Furthermore, its chemical properties are appropriate for the scaffold's formation due to its excellent biocompatibility. PEO is a non-toxic synthetic polymer since the by-product can be easily secreted by the renal and hepatic pathways [66]. Considering the biological properties, PEO itself has the ability to resist the protein adhesion and it will not interfere with the cellular functions or other target cells from the immune system which means PEO is having a low cytotoxic and material rejection incidence [66].

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter describes the materials, reagents, equipment, and instruments used in this study. Subsequently, the procedures of each experimental process are described in detail.

3.2 Materials

- Materials and Reagents
  - Hen eggshells
  - 10% sodium hypochlorite (NaOCl)
  - Distilled water
  - 1 M phosphoric acid
  - Polyethylene oxide (PEO) powder with M_w = 200 – 300 kDa
  - 316L Stainless Steel
  - Sandpaper No. 2000 and 2500
  - 0.3 and 1.0 μm aluminum oxide or alumina particles

- Research Equipment
  - 100 ml and 500 ml beakers
  - Stirring rod
  - Dropper
  - Micro spatula
  - 50 ml porcelain crucibles
  - Filter paper No.102
  - Magnetic bar

- Porcelain mortar and pestle
- Ziplock bag size 4x6 cm and 15x23 cm
- Square plastic basket
- Desiccator
- 1000 ml bottles
- Stirring bar

- Research Instrument
  - Analytical balance, Figure 10 - measures the weight of small samples and thus it is used for measuring the weight of reagents and samples [67].

Figure 10: Analytical Balance

  - Magnetic stirrer, Figure 11 - uses a magnetic force to rotate the stir bar placed inside the container of the liquid samples in order to stir or mix a solution. Therefore, it is used to mix calcium oxide and phosphoric acid to synthesize hydroxyapatite particles [68].

Figure 11: Magnetic stirrer

- The hot air oven, Figure 12 - generates hot air for removing moisture and controlling the temperature of materials and samples, which is used for drying the samples in this study [69].

Figure 12: Hot Air oven

- High-temperature furnace, Figure 13 - increases the temperature up to 1,200°C, which is used for high temperature treatment of the samples in this study, i.e. the conversion of calcium carbonate into calcium oxide.

Figure 13: High-temperature chamber furnace

- Vacuum filtration kit, Figure 14 - is a fast and convenient filtration kit using a vacuum pump to draw the liquid substance through the filter. Hence, it is used to filter the hydroxyapatite precipitates in this study [70].

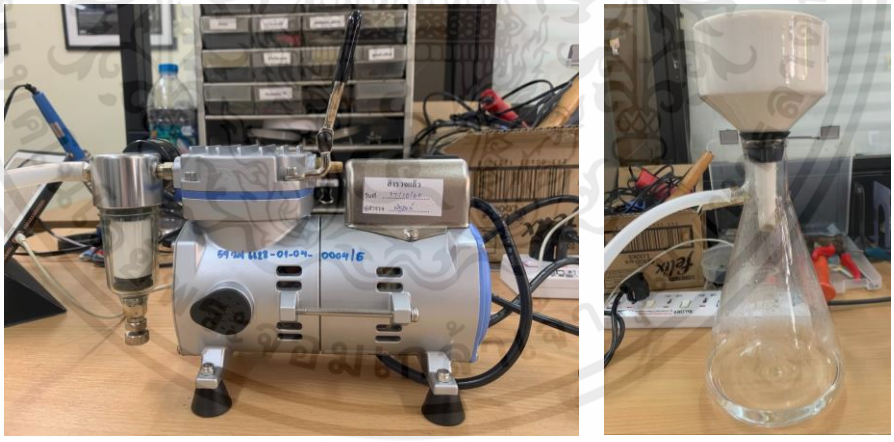

Figure 14: Filter holder sets with vacuum pump

- Thermogravimetric analyzer, Figure 15 - measures the weight of the samples whilst increasing the temperature. Hence. It could observe key changes in the material properties, including the degradation, decomposition, oxidation [71]. Furthermore, the by-products after the test can also be characterized in order

to analyze the chemical reactions happening during the test. Hence, this instrument is used to quantify the organic and inorganic component of the samples, as well as the transformation temperature from $CaCO_3$ into CaO.

Figure 15: Thermogravimetric analyzer [72]

- Fourier transform infrared spectroscope (FTIR), Figure 16 - is an analytical instrument used to identify functional groups of organic compounds. This technique measures the absorption of middle infrared radiation (IR) by the sample material versus the wavenumber ranging from 400 to 4000 cm^{-1}. Each wavenumber specifically corresponds to the stretching or bending of a covalent bond in the molecule, causing the changes in molecular dipole moment and IR absorption. Therefore, it is used to identify functional groups of calcium carbonate, calcium oxide, and hydroxyapatite in this study [73].

Figure 16: Fourier transform infrared spectroscope (FTIR) [74]

3.4 Preparing Eggshell Samples

Eggshells are collected from KMITL canteen and immersed into the water at room temperature to remove the surface contaminants and dirt, as shown in Figure 17.

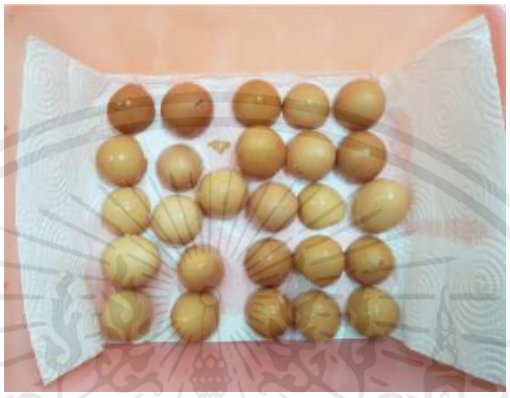

Figure 17: Prepared eggshells left to dry overnight.

After drying, they were ground into small pieces and immersed in the 25 ml of 10% sodium hypochlorite (NaOCl) solution to remove the organic components from the eggshells. After this process has been done, the eggshell samples should contain only inorganic components. However, as the immersion time was still remaining uncertain, the experiment was divided into 5 sets, each of which has different immersion time, including non-immersed samples, as shown in Figure 18. The purpose of this experiment was to find the suitable time for immersing the eggshell in NaOCl, which would lead to the minimum leftover organic components within the eggshells. The details of this experiment are explained below.

Figure 18: The eggshell samples were divided into 5 groups with different immersion time.

Set1: 8.587 g of eggshell were immersed in NaOCl for 30 minutes.

Set2: 8.536 g of eggshell were immersed in NaOCl for 15 minutes.

Set3: 8.582 g of eggshell were immersed in NaOCl for 10 minutes.

Set4: 8.627 g of eggshell were immersed in NaOCl for 5 minutes.

Set5: 1.198 g of eggshell were not immersed in NaOCl

After that, the samples were initially washed with tap water for 4 times and 1 time with distilled water to clean the immersed samples. The eggshell samples were brought into the oven to dry at 110˚C for 5 hrs., as shown in Figure 19.

Figure 19: Dry the eggshell in oven 110 degree Celsius.

After drying, samples were ground into powder, as shown in Figure 20, and the prepared samples were analyzed using TGA analysis.

Figure 20: The crushed samples in powder form.

3.5 Converting calcium carbonate ($CaCO_3$) into calcium oxide (CaO)

Raw eggshells are the sample rich in calcium carbonate ($CaCO_3$). In order to convert this kind of chemical component, heat treatment is brought to complete the process. At high temperature, calcium carbonate would decompose into calcium oxide (CaO) and carbon dioxide (CO_2) as shown in the chemical equation below:

$$CaCO_3 \longrightarrow CaO + CO_2$$

In this study 4 g of calcium carbonate powder per crucible was converted into CaO by using high-temperature furnace at 900˚C, as shown in Figure 21. To make sure that the process was complete, the samples were characterized by using FTIR analysis.

Figure 21: Convert powder of calcium by using High-Temperature Chamber Furnace

3.6 Converting calcium oxide (CaO) into hydroxyapatite (HA)

The process of forming HA can be done by first converting CaO into $Ca(OH)_2$ by adding 1 g of CaO powder into 125 ml of distill water and stirring by magnetic stirrer until becoming the homogenous suspension, as shown in the chemical reaction below. The pH after this step should be basic.

$$CaO + H_2O \longrightarrow Ca(OH)_2$$

After that, 10.8 ml of 1 M phosphoric acids were added into the solution and measured the pH of the mixed solution. The pH of this solution should be neutral because the mixtures are expected to yield the HA and water. The chemical reaction of this procedure is shown below:

$$10Ca(OH)_2 + H_2O \longrightarrow Ca_{10}(PO_4)6(OH)_2 + 18H_2O$$

From the equation, $Ca_{10}(PO_4)_6(OH)$ is known as hydroxyapatite (HA), which is the final product that we aimed to achieve. The main factor of this step is to control the molar ratio of calcium and phosphorus to be 1.67 as those found in theoretical HA. The next step is to leave the solution overnight for precipitation. The final product on the next day are

separated into 2 layers, which are water layer on the top and the precipitate layer on the bottom, as shown in Figure 22.

Figure 22: The precipitated sample after being left overnight.

The samples were stirred again using magnetic stirrer for 1 hour at room temperature at 900 rpm. The precipitates re-suspend into the liquid solution, which would be subsequently filtered, as shown in Figure 23.

Figure 23: Re-suspended solution before filtration.

The results appeared in the form of creamy-white samples which will then be dried in oven at 110˚C until the creamy-white samples turn into the white powder-like, as shown in Figure 24. The final step is to analyze the sample by using FTIR machine to confirm that the products are actually HA.

Figure 24 : Hydroxyapatite powder

3.7 Dip Coating Method

Dip coating is one of the effective techniques for the production of films on the material surface to improve the properties. The process involves the immersion of the samples into the solution. The thickness of the coated thin films depends on the density, viscosity, gravitational force, liquid-vapor surface tension, and dipping speed. If low-thickness films are required, dip coating should be done at slow speed, and *vice versa* [75, 76]. Moreover, the dip coat method can produce highly uniform films at room temperature using low-cost equipment [75, 76].

The dip coating process involved 3 main steps which are immersion, dwelling, and withdrawal, as shown in Figure 25.

1. Immersion: The clean and smooth substrate is immersed in the solution at a constant speed [77, 78].

2. Dwelling: The substrate remains immersed for a certain period of time to allow to the formation of films [77, 78].
3. Withdrawal: The substrate is withdrawn from the solution at a constant speed to achieve the uniform and smooth coated surface [77, 78].

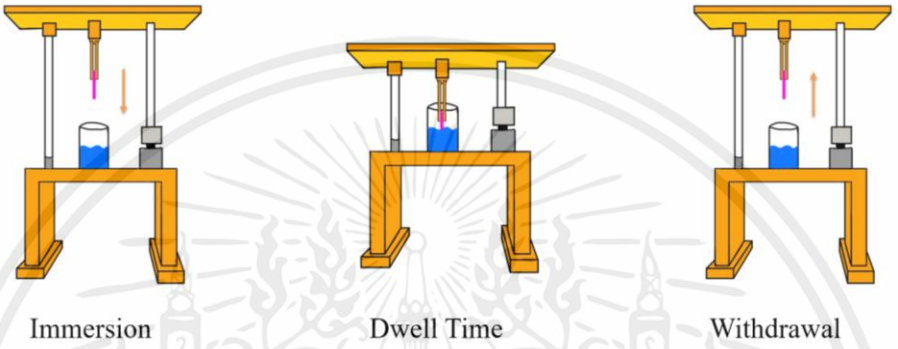

Figure 25: Steps of dip coat method

- Dip coating systems

The dip coater used in this study was manufactured by 3D printing, of which the size and geometry are shown in Figure 26, 27, and 28.

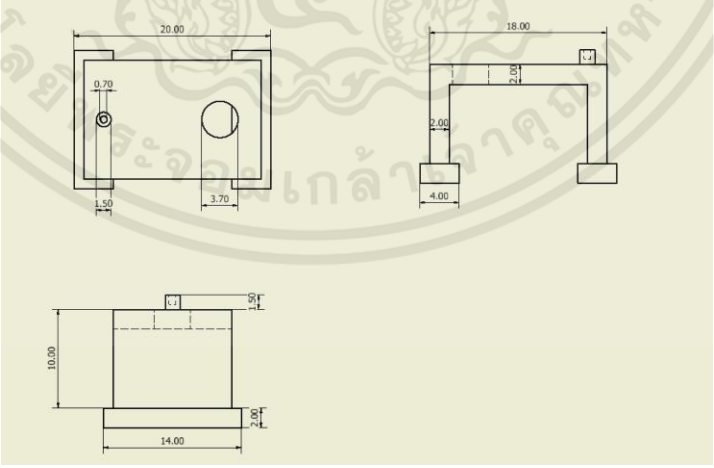

Figure 26: 2D dimension of base

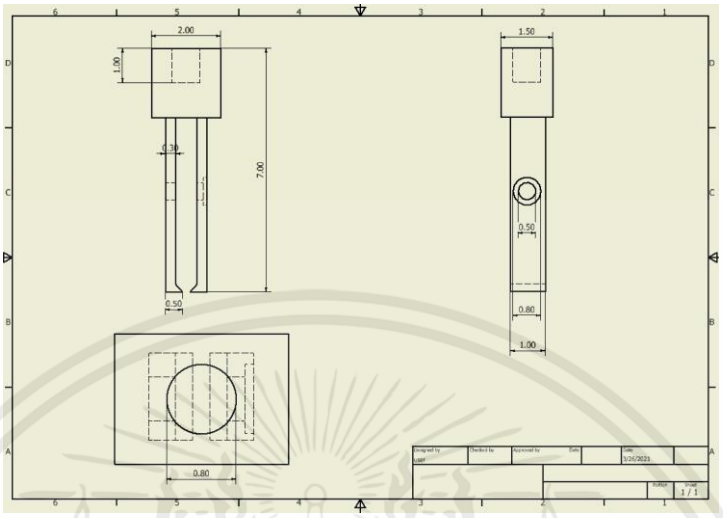

Figure 27: 2D dimension of Grip

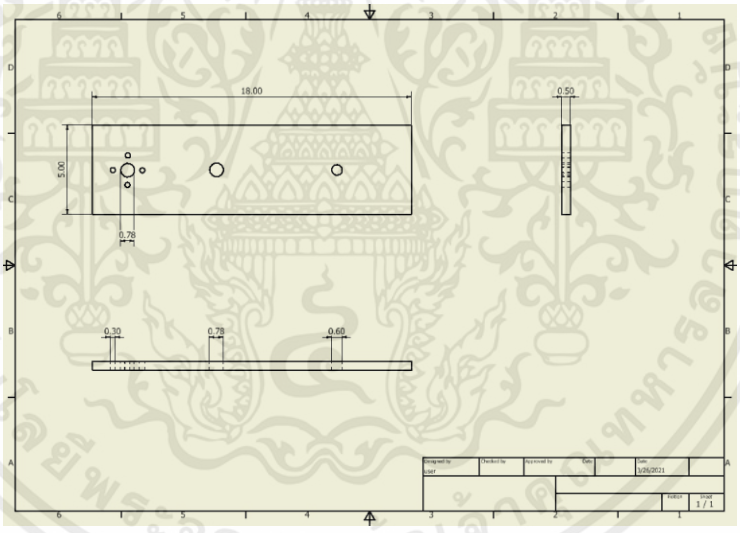

Figure 28: 2D Dimension of hold

- Dip coating process

  12% w/v of polyethylene oxide in distilled water solution was prepared as coating solution, as shown in Figure 29.

Figure 29: The preparation of polyethylene oxide in distilled water

After that, the 316L stainless steel has been cut into 2 x 5 cm size, as shown in Figure 30. The samples were then polished to acquire the smoother and clearer surfaces by first removing any dirt on the surface, then applying the sandpaper No. 2000 and 2500, respectively. The samples were finalized by polishing it again using 1.0 and 0.3 μm alumina powder, respectively.

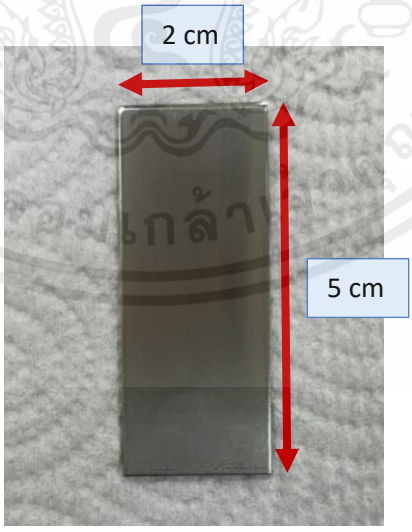

Figure 30: Polished 316L stainless steel 316 sample

HA powder was added into the PEO solution at 0.2%, 0.6%, and 1% w/v to be used as the solutions for HA/polymer composite film coating, as shown in Figure 31. The mixtures were stirred using magnetic stirrers. The dip coating process was done by submerging the sample into the solution using the custom-made dip coater, as shown in Figure 32. The immersion and withdrawal speed were 20.6 mm/s and the dwelling time was set at 3 minutes. The coated sample were left overnight at room temperature for drying.

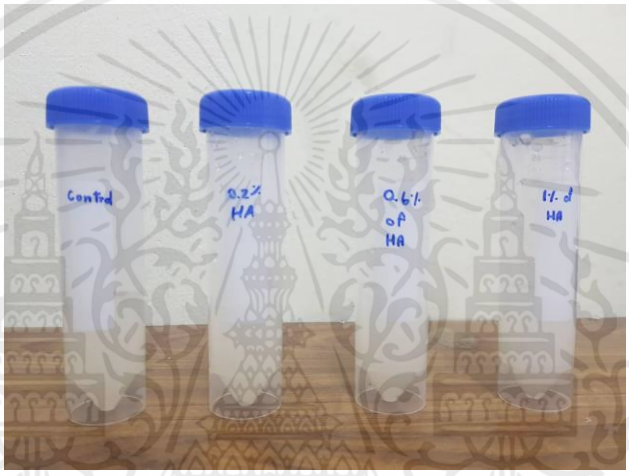

Figure 31: PEO/HA at various concentration.

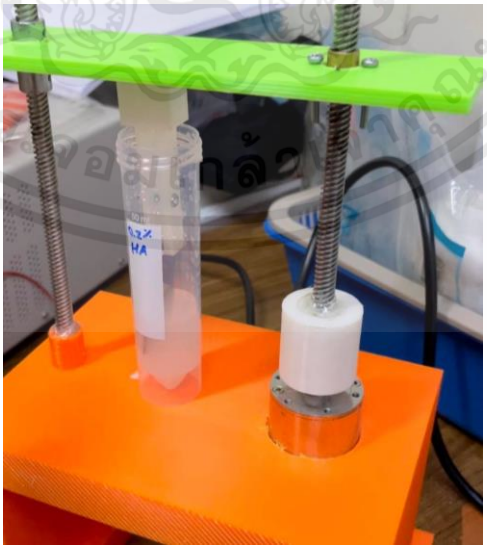

Figure 32: Apply the dwelling process for 3 minutes.

CHAPTER 4

EXPERIMENT RESULTS

4.1 Introduction

This chapter presents the results from the experiment carried out using the materials and methods described in the previous chapter. The first part would be focusing on the TGA results to find the suitable sample immersion time in NaOCl. The following part includes the FTIR analysis, which characterizes the chemical bonding within the material structure, to ensure that the material has undergone the expected reaction or transformation that leads to the HA formation. The final part of this project demonstrates the application of HA samples to form HA/polymer composite film on the biomedical 316L stainless steel by dip coating.

4.2 Analyzing TGA results

Figure 33 show the TGA results obtained from the eggshell samples immersed in the NaOCl solution at various immersion time have shown that the eggshells contain both organic and inorganic components. Moreover, the data also indicate the decomposition of $CaCO_3$ into CaO. The presented data are the weight change of the samples in relative to the initial weight, and thus the weight loss would indicate degradation, decomposition, or evaporation of the samples. It could be seen that there were two weight loss incidents during the temperature increase from 300 to 600°C, and the second one is above 800°C.

It is expected that the first incident was due to the degradation of organic component, whereas the second one was the $CaCO_3$ decomposition into CaO [79]. Hence, the leftover organic content and the final CaO yield could be calculated from the weight loss, of which the latter was around 45% of the initial weight. In terms of the leftover organic component, we focused on the remaining sample weight between 400 and 600°C as it is thought that all of the organic components have already been degraded, as shown in

Figure 34. By comparing with the initial weight, the leftover content of organic component could be calculated, the data are shown in Table 5.

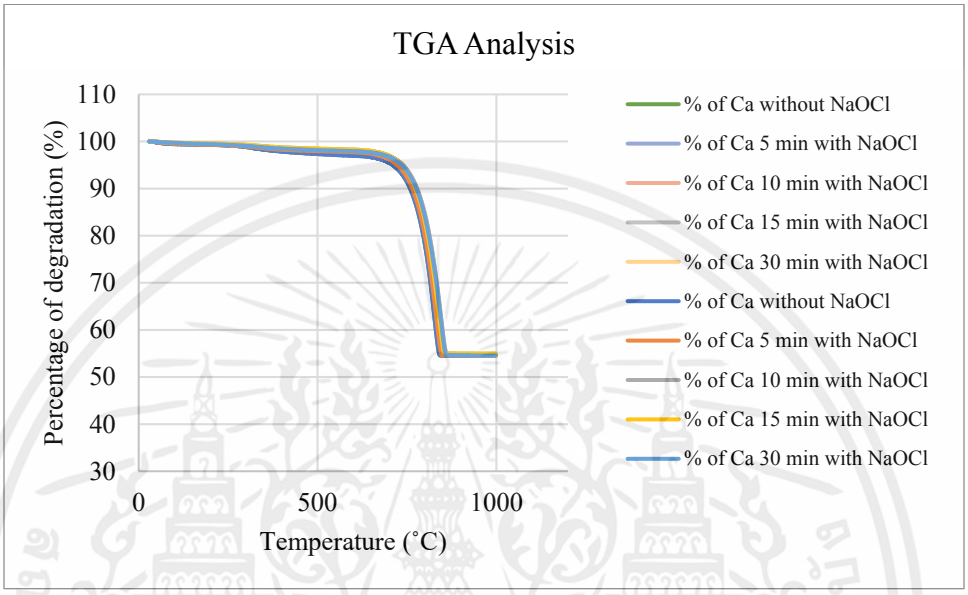

Figure 33: TGA analysis of the samples

[X-axis: Temperature (˚C), Y-axis: Percentage of degradation (%)]

From the Figure 34, there are 5 lines of the TGA results obtained during process of analyzing the percentage of organic compounds degradation. The value can be read out at 100% of mass originally for every individual line, while the declination of the slopes is shown through the increasing in temperature until it reaches around 50% of the total mass.

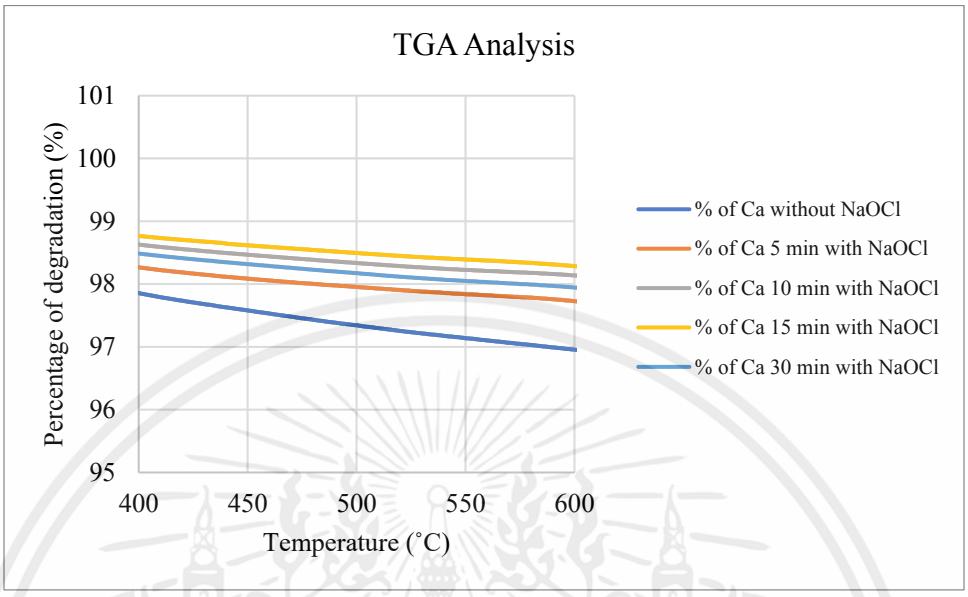

Figure 34: TGA Analysis at 400-600˚C

[X-axis: Temperature (˚C), Y-axis: Percentage of degradation (%)]

According to the TGA results, this can be illustrated in the percentage of protein loss as will be shown in the table below.

Table 5: Protein degradation at different time varying during immersing in the NaOCl

Eggshell Samples	**Degradation of Organic Component (%)**	**Remaining CaO (%)**
5 minutes in NaOCl	1.9069	45.4229
10 minutes in NaOCl	1.5293	45.1482
15 minutes in NaOCl	1.3776	45.0117
30 minutes in NaOCl	1.6797	45.4148
Not immersing in NaOCl	2.4167	45.4514

It could be seen from the results that the immersion of in NaOCl of 15 minutes was optimal for removing organic components as it provides the least leftover content. Moreover, increasing the immersion in NaOCl to 30 minutes did not provide lower organic

components than 15 minutes. Therefore, 15 minutes of NaOCl immersion was used throughout the rest of this study.

4.3 Analyzing FTIR results

As mentioned in the introduction of this chapter, FTIR was used to characterize the chemical bonds within the material structure. These data could be used for identifying the material type and its chemical structure. In this study, the data are divided into two parts: the $CaCO_3$ transformation into CaO; and the formation of HA.

- **Formation of Calcium carbonate ($CaCO_3$)**

According to the Figure 35, the existence of calcium carbonate ($CaCO_3$) in raw eggshell can be confirmed by using the FTIR analysis. Since there is the presence of water at 3648 cm^{-1} which refers to the O-H stretching, while the presence of carbonate group is at 1415 cm^{-1} and 874 cm^{-1} which refers to the C-O stretching and C-O bending, respectively [80, 81]. The presence of these functional groups show that the major components of the given eggshell samples contain $CaCO_3$.

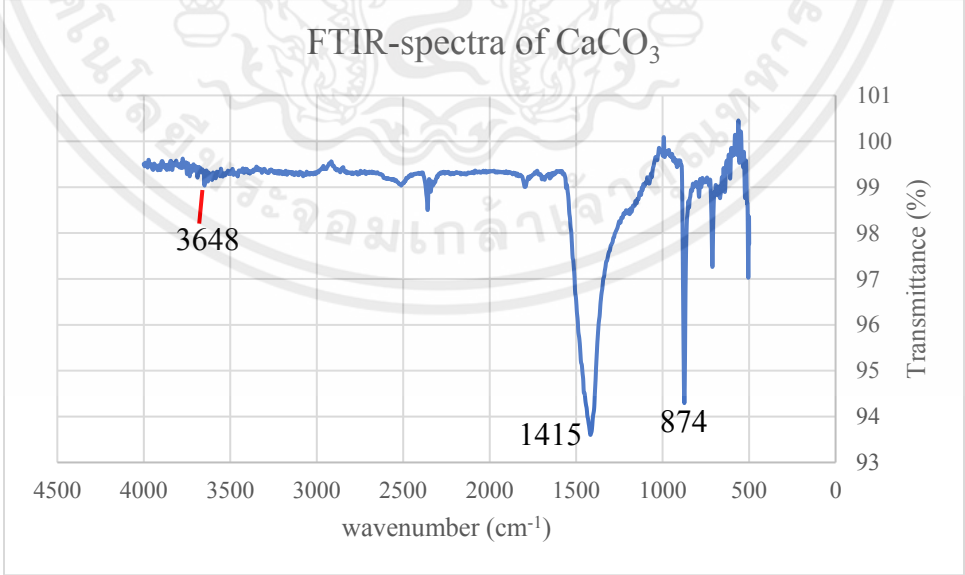

Figure 35: FTIR-spectra of eggshell samples after NaOCl immersion.

- **Converting $CaCO_3$ into CaO (Heat Treatment)**

The FTIR spectrum of the eggshell samples after heat treatment at 900°C is shown in Figure 36. It could be seen that there are 3 dominant peaks occur in the FTIR results. Each peak lies at 1057, 1463, and 3641 cm^{-1} of wavenumbers on the x-axis, while the y-axis refers to the percentage of transmittance of the samples. The exhibited peak occurring at 1057 cm^{-1} refers to C-O stretching of the primary alcohol, while at 1463 and 3641 cm^{-1} peaks refer to the O-H bending and O-H stretching respectively [80, 81]. These show the main functional group of the calcium oxide (CaO) obtaining from the raw eggshell [80, 81].

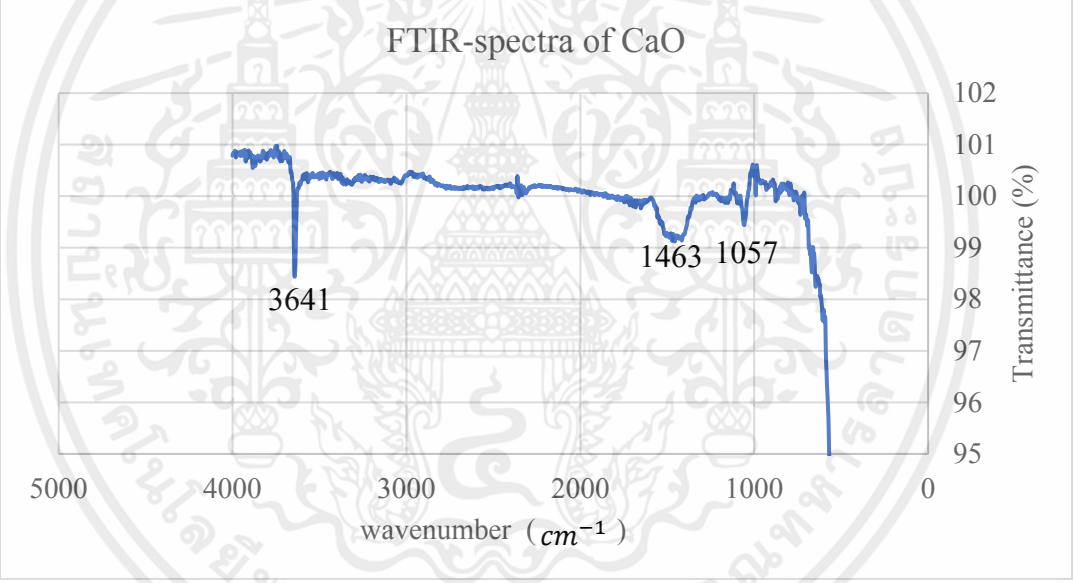

Figure 36: FTIR-spectra of eggshell samples after heat treatment at 900°C

From Figure 35, there are 3 dominant peaks occur in the FTIR results of CaO sample. Each peak lies at 1057, 1463, and 3641 $cm^{-1}$of wavenumbers on the x-axis, while the y-axis refers to the percentage of transmittance of the samples.

The FTIR-spectra of CaO can be explained as the ability of infrared that can penetrate through the samples. It can be seen that the peak occurring at 1057 cm^{-1} refers to C-O stretching of the primary alcohol, while at 1463 and 3641 cm^{-1} peaks refer to the O-H

bending and O-H stretching respectively. These show the main functional group of the calcium oxide (CaO) obtaining from the raw eggshell.

- **Converting CaO into HA**

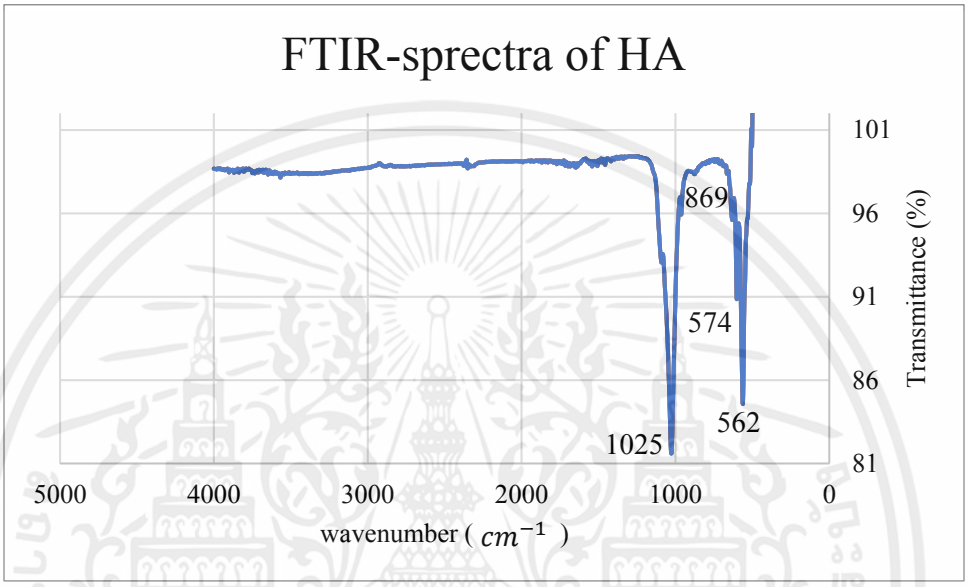

Figure 37: FTIR-spectra of eggshell samples after final reactions

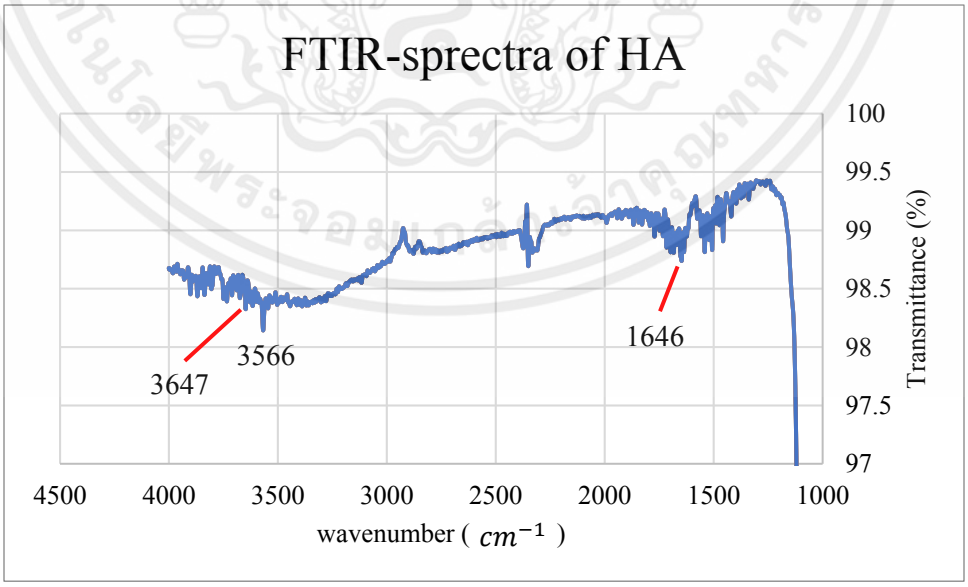

Figure 38: FTIR-spectra of eggshell samples after final reactions at 1000-4500 cm^{-1}

4.4 PEO/HA-coated on Stainless Steel

The dip coat process allows the solutions to be deposited onto the samples, the results can be observed differently according to the variation of the HA concentration. According to Figure 37(a), the coated layer of PEO without HA is clear and smooth due to the texture of purely polyethylene oxide, while the sample in Figure 37(b.) is having a white particle deposited on the certain area of the coated area, which is the sample with 0.2% w/v of HA. Figure 37(c.) and (d.) represent the samples of the coated layer at 0.6% and 1% w/v HA, respectively. It can be seen that at 0.6% concentration has more deposited particles in comparison with the 1% concentration. While the sample from 1% concentration was smoother as it did not have any crack on the coated layer as well as the deposited particles tend to spread thoroughly throughout the coated area.

It can be seen from the results that the dip-coated film was homogeneous. However, the HA-added films were noticeably different. Even though at 1% HA concentration tends to produce the nicely coated layer, the HA particles were not completely fused together. Hence, the HA particles could still move due to the gravity, resulting in inhomogeneous film formation. Although it would need further experiment to optimize the dip coating process from this study, it can be confirmed that HA particles have potential to be used for fabricating the HA/polymer composite film on the biomedical alloys. It is believed that the findings from this study could be beneficial for the future studies on the applications of biomedical materials.

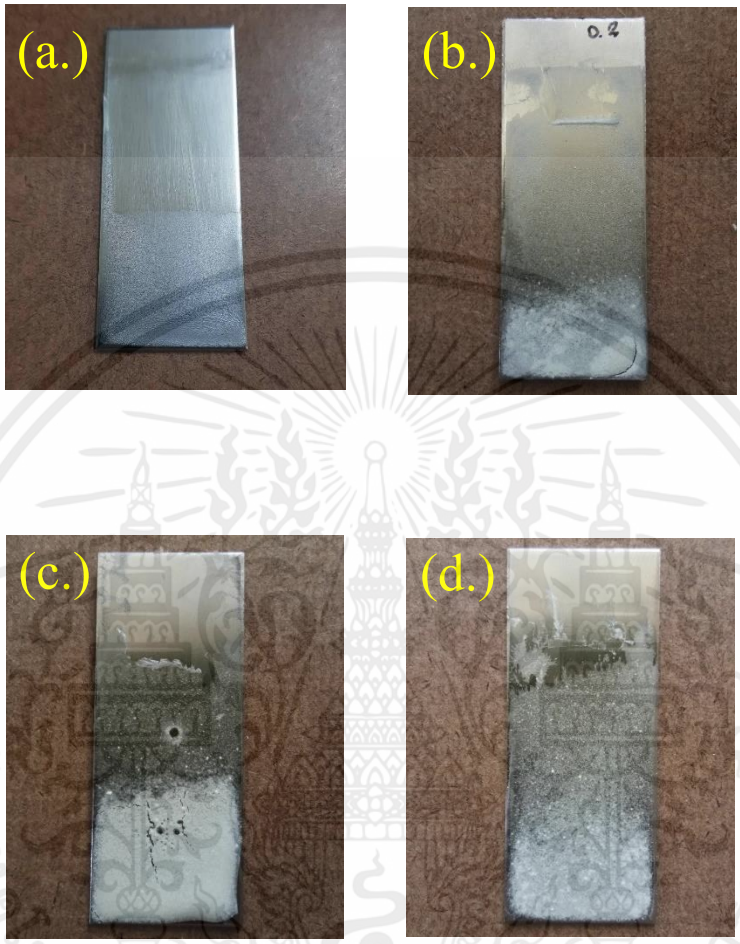

Figure 39: (a.) coated layer of non-added HA, (b.) coated layer of added 0.2(%w/v) HA, (c.) coated layer of added 0.6(%w/v) HA, and (d.) coated layer of added 1(%w/v) HA.

CHAPTER 5

CONCLUSION

5.1 Conclusion

The extraction of the hydroxyapatite is the process that use both chemical and thermal approaches to convert raw materials into the final product. It has been shown in this study that the hen eggshell can be processed into the pure hydroxyapatite using the method suggested in the literature. The TGA results show that immersion in 10% NaOCl for 15 minutes was sufficient for removing the organic components from the eggshells before proceeding to the following step. Heat treatment of the samples at 900°C decompose the samples into CaO with 45% yield, based on the TGA and FTIR results. Following the reactions with 1.0 M phosphoric acid, CaO was transformed into hydroxyapatite (HA), which exhibited hydroxyl group (OH^-) and phosphate group (PO_4^{3-}) from the FTIR spectra.

Hydroxyapatite powder has been further applied in dip coating process, which is commonly used for biomedical applications. It is shown in this study that 12% w/v PEO was mixed with HA powder could be used as dip coating solution to fabricate HA/polymer composite film on the surface of biomedical 316L stainless steel. However, it would need further investigation to improve the film morphology and properties.

5.2 Suggestion

Even though this study could reproduce the findings from the published articles, it would be worth investigating further in terms of the role of experimental parameters in order to gain better understandings on the rationale behind each process during the experiment, which would enable the further development of novel biomedical materials.

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APPENDICES

Code of dip coating

```
#define START 1 //
#define STOP 0


void setup()
{
 //Robojax.com L298N Stepper Speed STLPB-01
  // initialize the serial port:
  Serial.begin(9600);
  pinMode(ForwardPin, INPUT_PULLUP);
  pinMode(StopPin,INPUT_PULLUP);
  pinMode(BackwardPin,INPUT_PULLUP);
  //attachInterrupt(digitalPinToInterrupt(stopPB), stopMotor, FALLING);
}
void loop()
{
  if (digitalRead(ForwardPin)==HIGH){

  digitalWrite(in1, HIGH);
  digitalWrite(in2, LOW);
  // set speed to 200 out of possible range 0~255
  analogWrite(enA, 125);

  }


     if (digitalRead(BackwardPin)==HIGH){
    // now change motor directions
    digitalWrite(in1, LOW);
    digitalWrite(in2, HIGH);
    analogWrite(enA, 100);

    }
   }

   void stopMotor()
   {
     //Robojax.com L298N Stepper Speed STLPB-01
     if (digitalRead(StopPin)==HIGH)
     {
     digitalWrite(in1, LOW);
     digitalWrite(in2, HIGH);
     }
   }
```