EXTRACTION AND DEGRADATION RATE ANALAYSIS OF CALCIUM PHOSPHATE FROM DIVERSE FISH BONES: A COMPARATIVE STUDY

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Lemana CHEIKH

Thesis Advisors
Assist. Prof. Dr. İsmail Seçkin ÇARDAKLI
Assist. Prof. Dr. Ammar ZIDANOĞLU

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Thesis Advisors:
Assist. Prof. Dr. Ismail Seçkin ÇARDAKLI
Assist. Prof. Dr. Ammar ZIDANOĞLU

T.C.
Karabuk University
Institute of Graduate Programs
Department of Biomedical Engineering
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I certify that in my opinion the thesis submitted by Lemana CHEIKH titled "EXT RACTION AND DEGRADATION RATE ANALYSIS OF CALCIUM PHOSPHATE FROM DIVERSE FISH BONES: A COMPARATIVE STUDY " is fully adequate in scope and in quality as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Ismail Seçkin ÇARDAKLI .....

Thesis Advisor, Department of Metallurgical and Materials Engineering

Assist. Prof. Dr. Ammar ZIDANOĞLU .....

Second Advisor, Department of Biomedical Engineering

This thesis is accepted by the examining committee with a unanimous vote in the Department of Biomedical Engineering as a Master of Science thesis. 22/01/2024

Examining Committee Members (institutions)	Signature
Chairman : Assist. Prof. Dr. Ismail SEÇKIN (ATAU)	.....
Member : Assist. Prof. Dr. Ammar ZIDANOĞLU (KBU)	.....
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Member : Assoc. Prof. Dr. Daver ALI (KBU)	.....
Member : Prof. Dr. Aydın RÜŞEN (KMU)	.....

The degree of Master of Science by the thesis submitted is approved by the Administrative Board of the institute of Graduate Programs, Karabuk University.

Assoc. Prof. Dr. Zeynep ÖZCAN .....

Director of The Graduate Education Institute

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Lemana CHEIKH

ABSTRACT

M. Sc. Thesis

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Lemana CHEIKH

Karabuk University
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Thesis Advisors:
Assist. Prof. Dr. Ismail Seçkin ÇARDAKLI
Assist. Prof. Dr. Ammar ZIDANOĞLU
January 2024, pages 37

Converting waste fish bones into bioactive materials presents an innovative and eco-friendly approach to materials science. Fish bones, often discarded as waste in the seafood industry, are rich in calcium and phosphorous, making them ideal precursors for Calcium phosphate (CaP). in this study, different kinds of CaP materials were successfully extracted from diverse fish bone types like Carp fish (CF), Atlantic bonito (AB) and Gilt-head bream (GB) using a heat treatment method. The extracted white powders were characterized using X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FTIR), Transmission electron microscopy (TEM), and inductively coupled plasma mass spectrometry (ICP-MS) techniques and the outcomes compared the most popular phase of CaP material like beta-tricalcium phosphate (β-TCP) and hydroxyapatite (HA) which they are synthesized using microwave refluxing equipment. The XRD pattern of the CF sample

closely corresponded with the HA phase, while the AB and GB samples aligned with the biphasic calcium phosphate (HA/β-TCP) phase. The FTIR spectra analysis identified the presence of phosphate, hydroxyl, and carbonate groups. The XPS spectra determined the Ca/P ratio and the presence of trace elements such as C, Na, Mg, Si, and S. in vitro degradation studies were performed in phosphate-buffered saline (PBS) at 37 ºC over 1, 3, 7, and 14 days. The ion release profiles of calcium (Ca) and phosphorus (P) were monitored, revealing the most significant degradation rate occurred between 7 and 14 days. The highest leaching levels of Ca and P ions were observed in the Atlantic bonito fish, with concentrations reaching approximately 87.35 g/L and 78.13 g/L, respectively, after 14 days of immersion. Based on the obtained results, it is concluded that the biphasic calcium phosphate derived from Atlantic bonito, along with its content of Ca, P, and other minerals, exhibits a higher degradation rate compared to other samples. This indicates its potential as a promising bioactive material suitable for use as a bone tissue substitute.

Key words : Fish bones, Calcium phosphate, Characterizations, in vitro degradation rate.

Science Code : 92503

ÖZET

Yüksek Lisans Tezi

FARKLI BALIK KEMIKLERINDEN KALSIYUM FOSFAT EKSTRAKSIYONU VE BOZULMA HIZI ANALIZI: KARŞILAŞTIRMALI BIR ÇALIŞMA

Lemana CHEIKH

Karabük Üniversitesi
Lisansüstü Eğitim Enstitüsü
Biyomedikal Mühendisliği Anabilim Dalı

Tez Danışmanları:
Dr. Öğr. Üyesi Ismail Seçkin ÇARDAKLI
Dr. Öğr. Üyesi Ammar ZIDANOĞLU
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Atık balık kemiklerinin biyoaktif malzemelere dönüştürülmesi, malzeme bilimine yenilikçi ve çevre dostu bir yaklaşım sunmaktadır. Genellikle deniz ürünleri endüstrisinde atık olarak atılan balık kılçıkları, kalsiyum ve fosfor açısından zengindir, bu da onları kalsiyum fosfat (CaP) için ideal öncüler yapar. Bu çalışmada, Sazan balığı (CF), Atlantik palamutu (AB) ve Yaldızlı çipura (GB) gibi farklı balık kılçığı türlerinden ısıl işlem yöntemi kullanılarak farklı türde CaP malzemeleri başarılı bir şekilde ekstrakte edilmiştir. Ekstrakte edilen beyaz tozlar X-ışını kırınımı (XRD), X-ışını fotoelektron spektroskopisi (XPS), Fourier dönüşümü kızılötesi (FTIR), Transmisyon elektron mikroskobu (TEM) ve Indüktif olarak eşleşmiş plazma kütle spektrometrisi (ICP-MS) teknikleri kullanılarak karakterize edildi ve sonuçlar mikrodalga geri akış ekipmanı kullanılarak

sentezlenen CaP malzemesinin en popüler fazları olan beta-trikalsiyum fosfat (β-TCP) ve hidroksiapatit (HA) ile karşılaştırıldı. CF numunesinin XRD paterni HA fazına yakından karşılık gelirken, AB ve GB numuneleri iki fazlı kalsiyum fosfat (HA/β-TCP) fazıyla aynı hizadaydı. FTIR spektrum analizi fosfat, hidroksil ve karbonat gruplarının varlığını tanımladı. XPS spektrumları, Ca/P oranını ve C, Na, Mg, Si ve S gibi eser elementlerin varlığını belirledi. in vitro bozunma çalışmaları, fosfat tamponlu salinde (PBS) 37 °C'de 1, 3, 7 ve 14 gün yapıldı. Kalsiyum(Ca) ve fosforun (P) iyon salınım profilleri izlendi ve en önemli bozunma oranının 7 ila 14 gün arasında meydana geldiği ortaya çıktı. Ca ve P iyonlarının en yüksek sızıntı seviyeleri Atlantik palamut balıklarında gözlendi; konsantrasyonlar, 14 günlük daldırma sonrasında sırasıyla yaklaşık 87,35 g/L ve 78,13 g/L'ye ulaştı . Elde edilen sonuçlara göre Atlantik palamutundan elde edilen bifazik kalsiyum fosfatın Ca, P ve diğer mineral içeriğiyle birlikte diğer örneklere göre daha yüksek bir bozunma oranı sergilediği sonucuna varılmıştır. Bu, kemik dokusu yerine kullanılmaya uygun, umut verici bir biyoaktif malzeme olma potansiyelini gösterir.

Anahtar Kelimeler : Balık kılçığı, Kalsiyum fosfat, Karakterizasyonlar, In vitro bozunma oranı.

Bilim kodu **:** 92503

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SYMBOLS AND ABBREVIATIONS

SYMBOLS

° : Degree

°C : The degree Celsius

Å : Angstrom

a-axis : lattice parameter

b-axis : lattice parameter

c-axis : lattice parameter

cm : Centimeter

eV : Electronvolt

g : Gram

h : Hour

min : minute

mL : milliliter

nm : Nanometer

ppm : parts pert million

α : Alpha, cell angle

β : Beta, cell angle

γ : Gamma, cell angle

2θ : The diffraction angle

R3c : Rhombohedral space group

ABBREVIATIONS

AB : Atlantic bonito

BMD : Bone mineral density

C : Carbon

Ca : Calcium

CF : Carp fish

FTIR : Fourier transform infrared

GB : Gilt-head bream

HA : Hydroxyapatite

ICP-MS : Inductively coupled plasma mass spectrometry

Mg : Magnesium

Na : Sodium

OP : Osteoporosis

P : phosphorus

PMOP : Postmenopausal osteoporosis

PBS : Phosphate-buffered saline

S : Sulfur

Si : Silicon

TEM : Transmission electron microscopy

UV : Ultraviolet radiation

XRD : X-ray diffraction

XPS : X-ray photoelectron spectroscopy

PART 1

INTRODUCTION

1.1. BONE TISSUE

Bone tissues are recognized as connective and heterogeneous tissues composed of inorganic and organic parts in addition to water. Bone tissues are classified as cortical bone, with 80% of total bone mass consisting of hydroxyapatite (HA), collagen, and water. It protects the inner space and also prevents torsion and bending. At the same time, the spongy or cancellous bones with 20% of bones mass contain bone marrow and blood vessels [1, 2]. Bones serve essential functions to provide the structural framework for the body, protect internal organs, assist locomotion, maintain mineral homeostasis, and support blood cell production [3]. Bone cells are osteocytes, osteoblasts and osteoclasts. Where the osteocytes are cells that keep bone tissue alive and transport substances, and osteoblasts are responsible for the production of new bone throughout the process of bone remodelling; osteoclast are responsible for bone degradation to initiate normal bone remodelling and intervene in bone loss when the bones are exposed to pathologic conditions by expanding their resorptive action [4]. in this way, new tissues come to replace the aging parts.

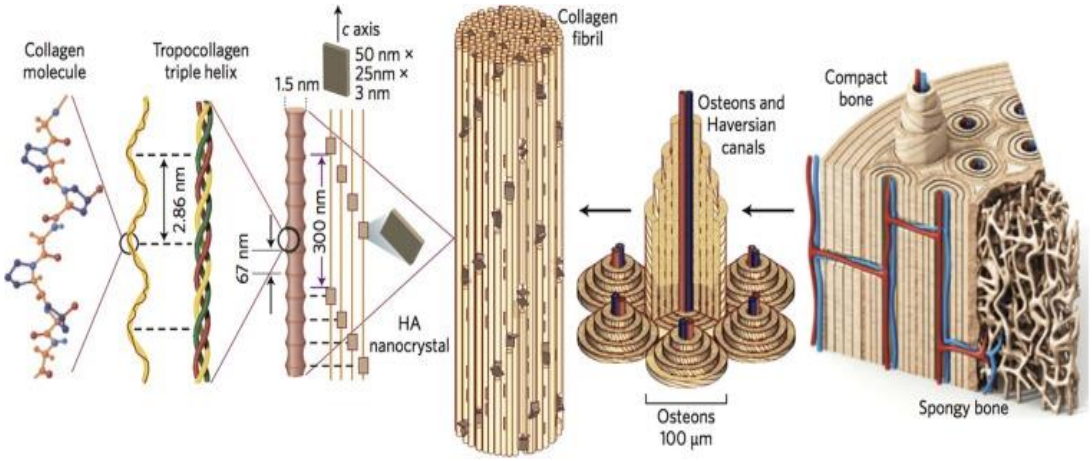

Figure 1.1. Hierarchical structure of the bone [5].

1.1.1. Bone Modelling and Remodelling

Bone modelling usually happens when bone resorption and formation occur on separate surfaces, meaning that the two processes are in opposition and are not connected. The length and diameter of the long bones develop continuously from infancy to adulthood. it is responsible for the skeleton's increased mass and morphological alterations [6].

Bone remodelling maintains bone mass and mainly affects adults. it is divided into five steps: (i) Activation occurs due to the influence of growth factors and cytokines. The former pre-osteoclasts are typically differentiated and stimulated into mature, active osteoclasts (ii) Resorption: this process breaks down the mineral matrix of old bone when a new bone has grown, and the medium has become acidic. (iii) Reflection: pre-osteoblast activation happens after osteoclast completion via apoptosis. (iv) Formation: Osteoblasts separate or differentiate, creating a new bone matrix. (v) Quiescence: osteoblasts now line the freshly made bone at this stage [6, 7].

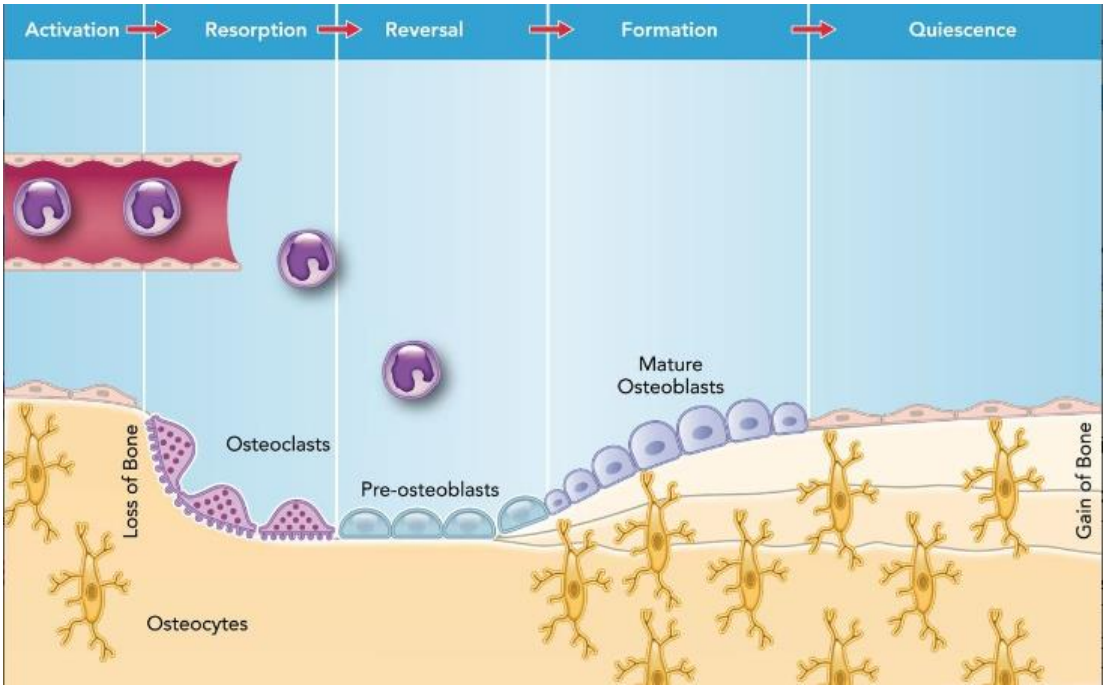

Figure 1.2. Bone remodelling process [8].

1.1.2. Bone Tissue Diseases

The human body is susceptible to various illnesses and injuries, particularly those affecting hard tissues like the hips, knees, and tendons. These conditions can sometimes produce disorders or dysfunctions that impact the bone and result in bone loss. Osteoporosis, Paget's disease osteomalacia, and other well-known conditions are examples of these disorders. OP is a metabolic condition or disorder that destroys the skeleton, causing bone mass loss, brittleness, and increased fracture risk. it frequently contributes to the high risk of fractures in older adults and persons of all ages because it gradually worsens over time until the patient has an abrupt shock, accident, or fall that breaks a bone. OP awareness is therefore becoming increasingly crucial and a reason to pay attention [9].

From an etiological perspective, primary and secondary OP are the two most frequent types of OP, according to the World Health Organization. OP usually affects people 50 years of age or older; primary OP is the result of changes associated with aging.

The postmenopausal decrease in estrogen secretion causes postmenopausal OP. Women may lose as much as 50% of their cortical and trabecular bone mass due to the postmenopausal process, compared to their peak bone mass, which occurs between the ages of 20 and 30. Conversely, several medical disorders and medications that reduce bone mineral density cause secondary OP [10, 12]. Joint pain, functional limitations, and spontaneous recovery are the hallmarks of a self-healing condition known as transient regional OP syndrome. This disease could show up as repeated attacks that could damage multiple joints, or it could show up as a single attack that affects one [13].

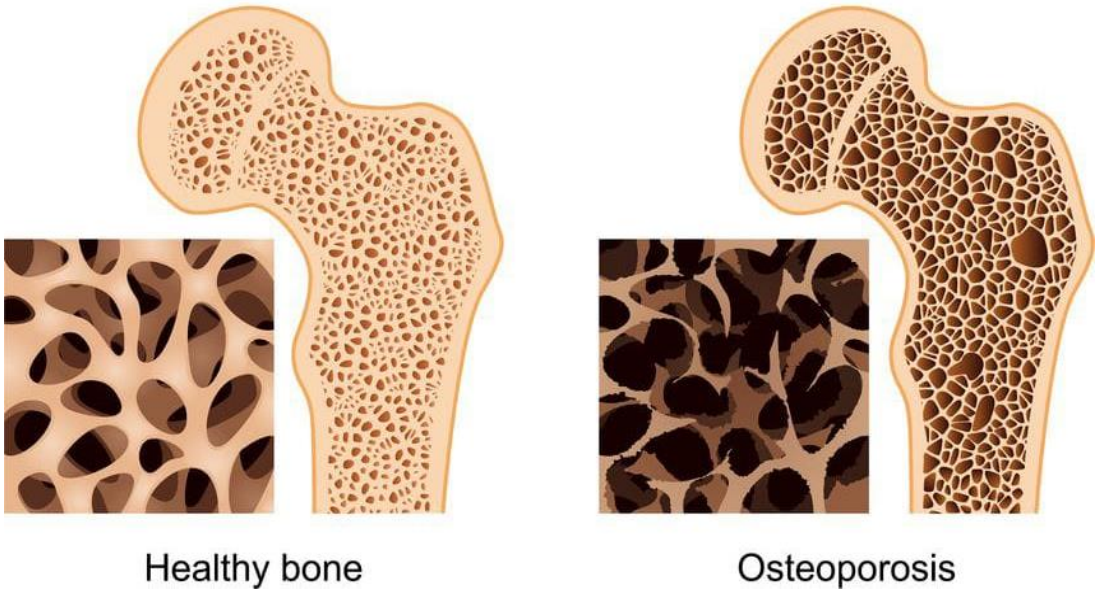

Figure 1.3. Microstructure of osteoporosis.

A problem that impairs the regulation of bone and bone muscle activity, leading to a focused shift in bone remodelling and dysfunction of bone formation, is Paget's disease of the bone, among other disorders that may affect the bone. Deformation and discomfort are the outcomes of the alteration in the characteristics of the bones, which may also lead to secondary OP and nerve compression. in 1886, James Paget made the initial diagnosis of Paget's disease, which was an unexplained multiple or unilateral osteopathy. After OP, it is the second acquired osteopathy that primarily affects older people, mostly men [14, 15].

1.1.3. Calcium Phosphate Biomaterials

Thanks to their beneficial properties, including biocompatibility, bioactivity, osteoconductivity, and bioresorbability, biomaterials are widely used in the medical disciplines of orthopedics, dentistry, and tissue engineering. The synthesis technique used strongly impacts the final properties of these CaP compounds and their effectiveness in medical applications [16]. Although synthetic materials possess numerous beneficial applications, especially in the biomedical field, several drawbacks warrant consideration. A primary disadvantage is the absence of minerals that characterize natural osseous apatite, which is not replicated in synthetic CaP [17]. Additionally, there are concerns regarding the use of chemical substances in their

production. The manufacturing process may contribute to an increased carbon footprint due to its high energy demands. Moreover, guaranteeing consistent quality in HA product samples is time-consuming and challenging [18].

Table 1.1. Examples of some biomaterials

Biomaterials	Characteristics	Advantages	Disadvantages	References
1-Calcium Phosphate	it has good compatibility and osteoconductivity properties	Good compatibility osteoconductivity reapsorbability	poor mechanical strength under continuous stress circumstances, low surface area	[19]
2- Bioglass	Excellent surface area, ability to induce apatite formation and cytocompatibility	Bioactive, resorbable, controllable degradation, good bone bonding affinity	Mechanical weakness Brittle, low fracture toughness	[20]
3- Collagen	biological properties and biodegradability	Biodegradable, biocompatible	lack of thermal stability low mechanical strength	[21]
4- Chitosan	natural polysaccharide, antibacterial and bioadhesive properties.	Antibacterial, wound healing.	Brittle, weak stability and insolubility	[22]
5- Alginate	biocompatible and highly hydrophilic nature, it can be crosslinked	Easy to get gelation, Cross-linking, biocompatibility	Hard handling, limited stability in physiological conditions	[23]
6- Polycaprolactone (PCL)	Excellent crystallinity, biodegradable polymer	Good mechanical strength, Cross-linking	its hydrophobic nature leads to the poor response of cells.	[24]
7- Metal scaffold	Biocompatible, Good mechanical properties that include mechanical strength and toughness	Good mechanical properties and biocompatibility	Non-biodegradable, besides toxic metallic ions, may cause allergic and inflammation reactions of the body.	[25]

1.2. BONE GRAFT

Bone replacements were first documented in 1682 when a dog's cranial bone graft effectively cured a cranial bone deficiency [26]. Recent developments in orthopedics and dentistry have resulted in a significant rise in demand for these alternatives. The main goal of bone grafting operations, which are still difficult and frequently include complications, is to restore a patient's functional and aesthetic needs fully. Thus, effective treatment planning requires a thorough grasp of the intricate biomechanics of natural bone, in addition to a fundamental understanding of the bone grafts that are commercially accessible. Furthermore, these factors also shed light on how to create natural biomimetic scaffolds that precisely replicate the physiological structure of real bone [27, 28].

1.2.1. Autograft

Because of their unique combination of osteogenic, osteoconductive, and osteoinductive qualities, autogenous bone grafts have become the gold standard among the many biomaterials utilised in grafted bone regeneration [29]. But its inevitable disadvantages cannot be disregarded as morbidity the biggest influence is at the donor site and the requirement for subsequent surgery other factors include the restricted quantity that can be extracted and the erratic pace of resorption. A plethora of different solutions have been presented to address these shortcomings. Nevertheless, none of these alternatives has been able to fully replace the requirement for autogenous bone in all circumstances. Xenografts have the added drawback of not having osteogenic potential, while allografts are substantially more expensive. These negative aspects have limited their clinical use [30, 32].

1.2.2. Allograft

Bone allografts have been utilised as a natural alternative to heal skeletal defects for many years. Because their supply is less restricted, they enable skeletal restoration structurally and their surfaces encourage the formation of new bone, they present an alluring substitute for bone autograft. The need for allografts has increased

dramatically due to the growing number of revision arthroplasties performed on an ageing population and the recent trends in minimally invasive surgery, especially in the spine, where there is a rapidly growing need for bone grafts or substitutes. in recent years, there has been a notable increase in the amount of allograft available. it's the most popular alternative to bone in Europe. This is comparable to the circumstances previously noted in the USA, where an estimated 800,000 grafts are applied annually [33, 34].

Still, there are many questions about how safe any bone allograft is. Professional organizations and the European Union have recently released guidelines to optimise the quality and safety of allografts and eliminate donors who risk disease transmission [35].

1.2.3. Xenograft

Compared to human bone, the structure of bone tissue from other species is similar. These bones can be ideal substitutes for bone grafting because they exhibit osteoconductive and osteoinductive activities [36]. Alternatives like xenograft-derived bone scaffolds are appealing because they don't cause any patient morbidity during recovery, can be obtained in large quantities from healthy donors with regulated biological and mechanical characteristics and don't carry the risk of human disease transmission that comes with allografts. However, xenograft tissues risk triggering an unfavourable host response if they include residual chemicals applied during tissue processing, contaminating pathogens not eliminated during tissue processing, or immunologically active foreign cellular material. Orthopaedic applications have mostly abandoned xenograft bone transplantation due to its poor clinical outcomes in the past [37, 38].

1.3. PROBLEM STATEMENTS

Due to their excellent properties, calcium phosphate materials, pivotal in orthopedics and dentistry, have traditionally been synthesized through various processes. However, there is a growing trend towards extracting these materials from natural sources, like

fish bones. This innovative approach serves a dual purpose: it reduces waste and maximizes the use of available resources.

in the seafood industry, fish bones are often discarded, yet they are an abundant source of calcium and phosphorous – the key components of CaP. By redirecting these waste materials to extract CaP, there is an opportunity to address a major challenge in materials science: the sustainable utilization of waste. The extracted CaP, with its recognized bioactivity and potential in biomedical applications, particularly as bone tissue substitutes, represents a promising avenue for both environmental sustainability and advancements in bioactive material development.

1.4. OBJECTIVES OF THE THESIS

This study investigates the feasibility of converting different fish bones into various forms of CaP materials. Specifically, it examines the extraction and characterization of CaP from Carp fish (CF), Atlantic bonito (AB), and Gilt-head bream (GB) using a heat treatment method. The research compares these extracted materials with commonly synthesized CaP phases like beta-tricalcium phosphate (β-TCP) and HA. it also aims to assess these materials' properties and in vitro degradation behavior to evaluate their suitability and efficiency as bioactive materials for potential biomedical applications. The study's ultimate goal is to establish a sustainable approach to waste utilization while advancing the development of innovative materials for healthcare applications.

1.5. SIGNIFICANT OF THE STUDY

CaP materials plays a significant role in bone tissue engineering. The goal of this work is to create novel biomaterials. These materials are very promising for use in biomedical applications, particularly in the future in the biomedical industry. They are synthesised in a modest and inexpensive method that can be manufactured locally, reducing the high cost so that ordinary citizen can benefit when needed.

PART 2

LITERATURE REVIEW

2.1. CALCIUM PHOSPHATES

CaP minerals are made up of phosphate anions and calcium cations. They are recognised as the predominant inorganic component of about 60% of all native human bones. The first evidence of CaP in bones dates back to 1769 [39]. These substances have been classified into various groups. Synthetic CaP have been the subject of intensive clinical research since the 1900s. Since then, CaP based coating methods scaffolds, implants, bone cements, and other bone-regenerative applications have been developed and commercialised. Comparable properties of CaP have been investigated for applications related to bone regeneration [40]. in addition, CaP materials are considered attractive biomaterials due to their excellent biocompatibility and non toxicity of their chemical components. The most common are HA and TCP. These species are commonly used because of their crystalline structure, osteoconductive ability, and chemical composition, which are similar to skeletal tissue [41, 42].

Table 2.1. Structure of HA and β-TCP particles

Name	Formula	Ca/P	Symbol
Hydroxyapatite	Ca10 (PO4)6(OH)2	1.67	HA
β-tricalcium phosphate	Ca3(PO4)2	1.50	β-TCP

2.1.1. β-Tricalcium Phosphate

β-TCP, is a biodegradable ceramic that is used in a wider range of clinical applications it can be used alone or in combination with other calcium supplements or as a part of biphasic CPs in the form of macroporous granules, dense or scaffolds in the biomedical applications and to improve the regeneration

of bone tissue. Previous research has been applied to hip implants and treating craniofacial deformities as a femoral root coating [43]. The crystal structure in (Figure 2) indicates that β-TCP has a rhombohedral structure with a unit cell parameter of a=b=10.4352 Å and c=37.4029 Å, and α=β=90° γ=108.65°. This is consistent with the R3c space group of β-TCP. 3.07 g/cm3 is its density. Tetrahedral phosphate centers joined to calcium ions via oxygen form the complex structures of β-TCP.

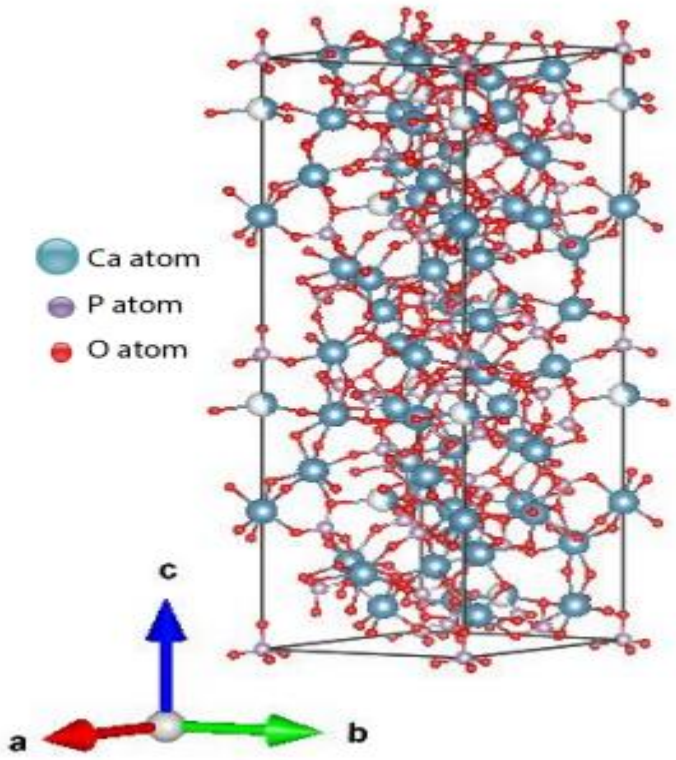

Figure 2.1. The unit cell structure of β-TCP.

2.1.2. Hydroxyapatite

HA [$Ca_{10}(PO_4)_6(OH)_2$] is an inorganic composite resemble the properties and chemical formula to those of main inorganic constituent of teeth and bones, due to it's high bioactivity nature, biocompatibility, porous structure and mechanical strength, become a preferred bone substitute material. Furthermore, it's used as a surface coating material, drug delivery systems and a scaffold material in the bone tissue regeneration [44]. According to a study by V. P. Orlovskii et al, HA is stable

towards bioresorption, has no negative effects on humans, and can form a strong link with bone tissue. it also shows osteoconductive behaviour. The chemical and phase composition, microstructure, pore size, and pore volume of HA ceramics are among the several variables that affect their biological behaviour [45].

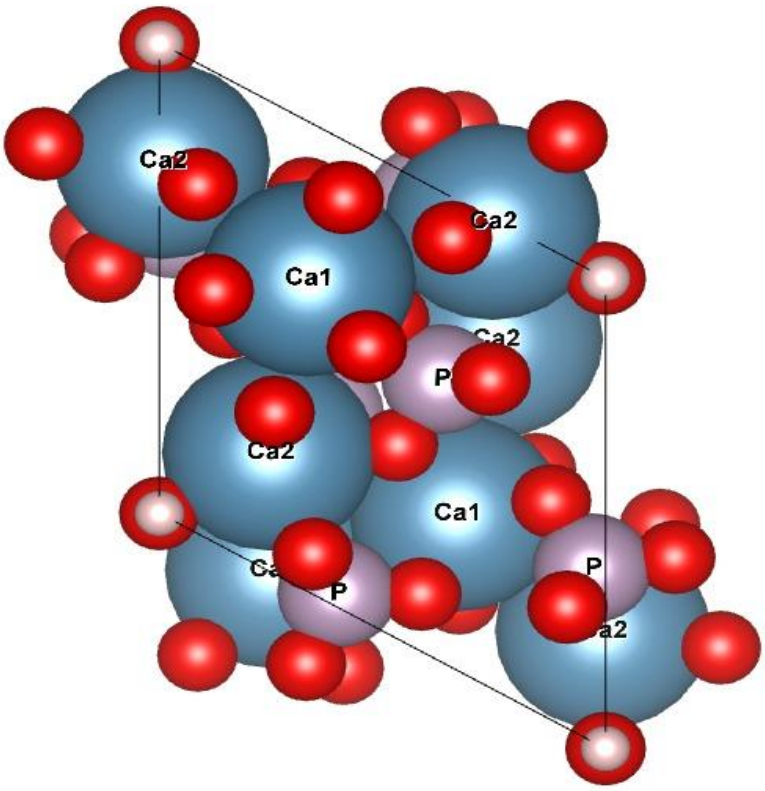

Figure 2.2. Structure of HA.

2.2. METHODS TO SYNTHESIZE B-TCP AND HA PARTICLES

Hydrothermal conversion or chemical precipitation are two methods for processing and synthesizing HA and β-TCP, two minerals essential in biological applications.

Wet chemical precipitation, sol-gel procedures, hydrothermal processes, and biomimetic strategies are other methods that could be used for the same goal.

2.2.1. Hydrothermal Method

With this technique, nanoparticles can be produced from ambient temperature to high temperatures. The morphology of the materials to be created is monitored under low pressure or high pressure settings, depending on the vapour pressure of the major components in the reaction hydrothermal synthesis has the benefit of producing materials with good crystallinity and homogeneous shape and size qualities at low temperatures.

2.2.2. Wet Precipitation Method

The wet precipitation process is one of the most common methods for making β-TCP powders. This technique creates weakly crystalline β-TCP by combining precursors based on $PO4^{3-}$ and Ca^{2+} in a neutral or basic solution. The main benefits of using wet precipitation methods are that they are simple to process, don't require complicated equipment, have low reaction times and temperatures, are inexpensive raw materials, have controllable particle morphology and mean size, are reproducible and scalable, can be used in industrial settings, and have easily controllable synthesis conditions.

2.2.3. Solgel Method

Now the sol-gel method is used to ascertain the functional properties of textile surfaces; more and more projects are utilising it every day and it is advised that this approach be utilised instead of others during the development process. Recent research has demonstrated the functional qualities of the sol-gel method, which are obtained by combining sols and gels including flammability, antibacterial protection, UV protection, and the ability to provide anti-crease effects.

The basic steps of the sol-gel method are typically as follows: A high-temperature process, polycondensation, gelation, ageing, drying, and precursor hydrolysis.

2.3. EXTRACTION OF CALCIUM PHOSPHATE FROM NATURAL SOURCES

Most commercial CaP ceramics are made from synthetic raw materials using various techniques, such as Hydrothermal, Wet precipitation method and solgel theqniue. Nevertheless costly reagent-grade chemicals are typically utilised in conjunction with intricate and time consuming procedures to prepare synthetic CaP ceramics [46, 48].

Natural sources can also be used to economically and easily extract HA and TCP, including sheep bovine, chicken and fish [49]. Which have positive effects on both environment and economy.

2.3.1. Bovine Sources

There is no denying the manufacturing and supply of meat have a significant social and economic impact. Nonetheless, their capacity to produce pollutants has led to global environmental concerns. To guarantee that the waste produced by this industry finds a proper and sustainable home, solutions are required. The majority of this waste is made up of cow bones, which can be reused to lessen environmental impact greatly.

HA, a promising biomaterial with enormous potential in many fields, is the primary mineral component of bone. Because of its biocompatibility and bioactivity, HA derived from cow bones has been used as a biomaterial in orthopaedic and dentistry fields. Dense HA bioceramics still don't provide enough qualities for applications that demand strong mechanical performance [50].

Furthermore, the benefits of naturally derived bovine bone derivative scaffolds for bone grafting applications encompass osteoconductivity, availability, affordability, biocompatibility and biodegradability. These benefits are associated with the inherent characteristics of natural bovine bone [51].

2.3.2. Chicken Sources

Chicken bone wastes of the food and agriculture industries is typically thrown away without being completely utilised. The conversion of chicken bone waste into CaP materials has not been widely documented, in contrast to other biogenic bones like fish or cow bones [52].

By 2030, egg production is predicted to increase gradually to about 90 million tonnes, in line with the growing demand for eggs from consumers around the globe [53]. As a result, there is also an increase in the amount of eggshell waste generated sadly this waste is typically disposed of in landfills, further polluting the environment. Given the circumstances, it will be more advantageous for the environment and the economy if the eggshell waste is transformed into worth while products. Eggshells are a cheap and easily accessible natural calcium source because of their high biomineral calcium content. About 94% of eggshell is made up of calcium carbonate, with trace amounts of magnesium, phosphorus, potassium, sodium and iron. Less than 1ppm of other microelements, including zinc, copper, iron, and manganese, can also be found in the eggshell. According to reports, these components are essential for enhancing osteoconductivity, osteoinductivity, and cell proliferation, which makes HA derived from eggshells biologically superior to HA derived from commercial reagents [54].

2.4. MARINE WASTE

Food security, the economy, aquatic and terrestrial environments are all impacted by problems with food waste, waste disposal, and by-product management [55]. About 91 million tonnes of fish and shellfish are consumed annually, and the fishing industry, which is expanding quickly, is a major contributor to the food industry [56]. Approximately 40–50% of the fish are by-products released as waste after processing [57]. Big fish processing by-products like fish bones are considered useless waste and impractical. To our knowledge, no accepted method for properly treating fish bones in the market has been agreed upon. As a result, fish bones are typically utilised to make animal meals near an animal feed production facility, albeit this

has limited advantages [56]. Many researchers have recently focused on evaluating fish bones as a source of CaP ceramics because they could yield a superior bioengineering material.

2.5. EXTRACTION OF CALCIUM PHOSPHATE FROM MARINE SOURCES

Natural CaP sources like fish bones offer minimal side effects, biocompatibility, and bioactivity. Human bone's mineral component is similar, reducing rejection. This eco-friendly method reduces waste and artificial methods, aligning with green chemistry and sustainability principles in the fish industry. Sustainable extraction of HA from fish bones was investigated by Dabiri, Rezaie, et al, who evaluated its chemical, morphological, and biocompatibility qualities. Compared to commercial HA, it showed better biocompatibility and nickel absorption from aqueous solutions. These results show that HA has promise as a green replacement in medicine and the environment [58]. Muhammad, Gao et al [59], employing an ionic liquid, effectively extracted HA from the fish scale debris and yielded 32%. The results of the characterization experiments verified HA's characteristics and great thermal stability. Cell viability studies on kidney cells (HEK) and human epidermoid carcinoma (A431) cells confirmed the isolated HA's biocompatibility. Challoob, Duaa et al. isolated biological HA from common carp fish bones, after heating the bones to 900°C, crystals of HA formed, containing phosphate and hydroxyl functional groups. The HA particles resembled little globes [60].

Table 2.2. The influence of trace elements on bone health and structure.

Element	Biological Effects
Magnesium	• Stimulate cell differentiation • Cell adhesion and enhanced bioactivity
Silicon	• improve osteogenic differentiation • Enhance mechanical property Enhance cell differentiation
Zinc	• improve the differentiation of osteogenic cells • Avoid osteoporosis • inhibit osteoclast cell formation • increase angiogenesis
Fluoride	• Enhance strength and corrosion resistance. • Stimulate osteoblast activity • Avoid osteoporosis

PART 3

MATERIALS AND METHODS

Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) (Merck-Germany) and di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) (Merck-Germany) were used as precursors to prepare HA and β-TCP materials. Sodium chloride (NaCl) (Merck Germany) and sodium hydrogen phosphate ($Na_2 HPO_4$) (Merck Germany), potassium chloride (KCl) (Merck Germany), and potassium dihydrogen phosphate (KH_2PO_4) (Merck Germany) were used to prepare a phosphate-buffered saline (PBS) solution.

3.1. SYNTHESIS OF B-TCP AND HA MATERIALS

To synthesize a pure phase of β-TCP, 0.9 moles of $Ca(NO_3)_2$ were dissolved in 200 mL of distilled water to create Solution A. Concurrently, 0.6 moles of $(NH_4)_2HPO_4$ were dissolved in another 200 mL of distilled water to form Solution B. Solution B was then gradually added to Solution A, with continuous stirring for 30 min. For β-TCP, the pH was adjusted to 7 by adding NH_4OH. This resulted in a white residue that was subsequently transferred to a microwave, specifically an ARCELIK MD 500 equipped with a reflux system, and microwaved for 5 minutes. For HA synthesis, the corresponding amounts of reagents are 1.0 mole of $Ca(NO_3)_2$ and the pH adjustment to 10, with a microwaving duration of 15 min. The resultant white precipitate was then washed, filtered, and dried in an oven at 80°C for 24 hours, followed by calcination at 1000°C for 2 h.

3.2. EXTRACTION OF HYDROXYAPATITES FROM FISH BONES

Fish bones from CF, AB, and GB were procured from markets in the Karabük area of Turkey. These bones underwent extensive cleaning: they were boiled at 100°C for 3 h to ensure thorough sterilization and then dried in an oven at 80°C for 24 h After drying,

the bones were crushed into smaller fragments and subjected to a heat treatment at 1000°C for 2 h, which according to previous studies have shown excellent results for CaP materials extracted from fish sources. This process resulted in a fine powder, which was further ground to achieve a consistent particle size, making it suitable for subsequent characterization and analysis.

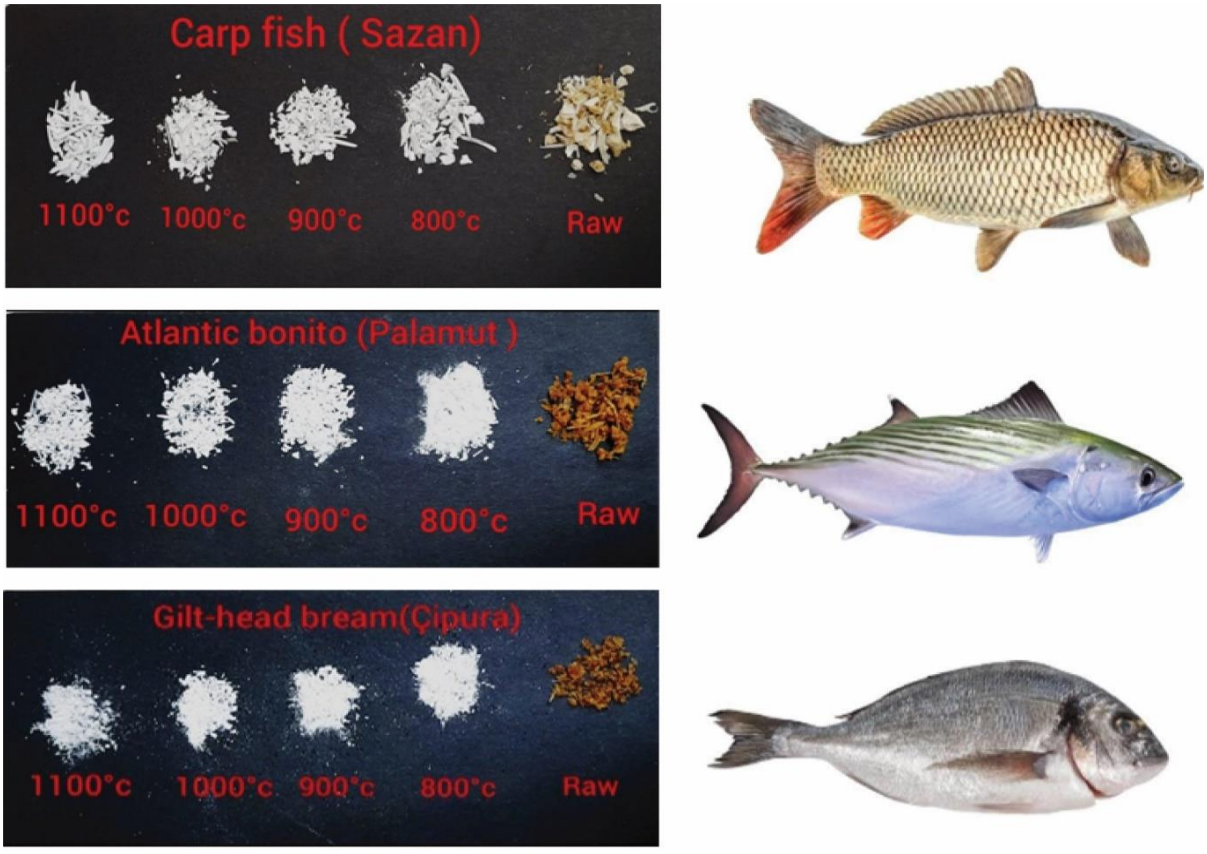

Figure 3.1. Shows the collected bones treated at different temperatures.

3.3. CHARACTERIZATION

The phase purity, crystallinity degree, and lattice parameters of the produced powders were measured using X-Ray Diffraction (XRD, Rigaku Ultima IV model). Steps of 0.03° were used to capture diffraction patterns in the 2 range of 20° to 80°. Fourier Transform infrared Spectroscopy (FTIR, Bruker IFS 66/S) was used to determine the vibration bands associated with functional groups in the materials. At normal temperature, scans were performed in the 4000 to 400 cm-1 wavelength range. Transmission electron microscopy (TEM, CM120, PEG, Philips) at 120 keV was used

to examine the generated particles of HA and β-TCP for morphology and characteristic particle size.

3.4. IN VITRO DEGRADATION ANALYSIS

Powder samples were tested in Phosphate-Buffered Saline (PBS, pH 7.4) to determine their degradation rate. The samples were stored in 15 mL of PBS at 37°C for 1, 3, 7, and 14 days. The samples were painstakingly removed at the end of each period, and the resulting filtrates were collected for further study. By using inductive coupled plasma mass spectrometry (Aglinet 7800 ICP-MS), we were able to ascertain the leaching patterns of calcium (Ca^{2+}) and phosphorus (P^{5+}) ions in the PBS medium. in addition, pH levels were monitored continuously to ensure accuracy using pH meter. Phosphate-buffered saline (PBS) solution was prepared by adding 800 ml of distilled water in a duran bottle, measured out and adding 8g of sodium chloride (NaCl) (Merck Germany) and 1.44g of sodium hydrogen phosphate ($Na_2 HPO_4$) (Merck Germany), then 0.2g of potassium chloride (KCl) (Merck Germany), and 0.24g of potassium dihydrogen phosphate ($KH_2 PO_4$)(Merck Germany). Bringing the pH to 7.4 using HCl, adding distilled water until the total volume reaches 1 litre, sterilizing in an autoclave on a liquid cycle, storing at room temperature.

Table 3.1. Required components for PBS preparation.

Component	**Amount**	**Concentration**
Sodium chloride (NaCl)	8g	0.137 M
Sodium hydrogen phosphate($Na_2 HPO_4$)	1.44g	0.01 M
Potassium dihydrogen phosphate ($KH_2 PO_4$)	0.24 g	0.0018 M
Potassium chloride (KCl)	0.2g	0.0027 M

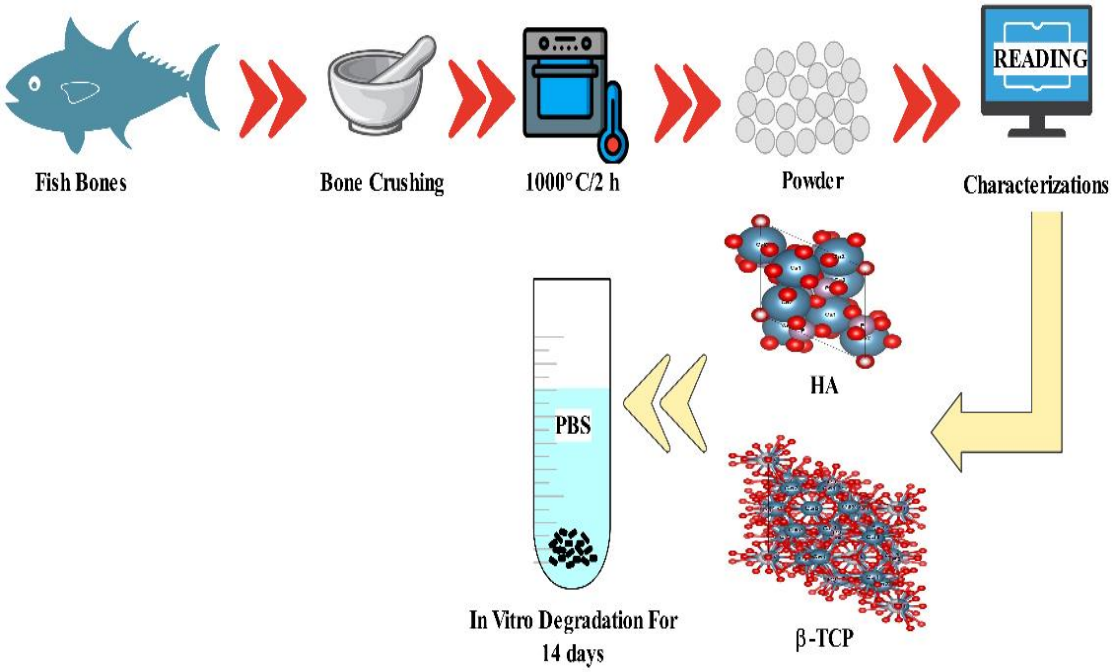

Figure 3.2. Experiment flow chart

PART 4

RESULTS AND DISCUSSION

4.1. STRUCTURAL CHARACTERIZATION OF HA, β-TCP, AND FISH-BONE SAMPLES

Wet precipitation-microwave refluxing system was used to prepare the pure phase of HA and β-TCP materials which they used as reference materials to evaluate the phases of the extracted materials. The X-ray diffraction patterns of synthetic and extracted materials are illustrated in Figure 4.1. The pure phase of HA was a good match with the standard phase of HA hexagonal framework (JCDPS No. 09-432). While the pure phase of β-TCP agreed with the standard phase (JCDPS No. 09-0169). The consistency of these findings with those of other studies provides further evidence for the reliability and repeatability of the synthesis methods used to produce HA and β-TCP. The XRD pattern of the CF identified as pure HA reveals interesting characteristics that are significant for its biological applications. The observed reduction in the lattice parameters, specifically the 'a' and 'c' axes, along with a decrease in crystallite size and degree of crystallinity, presents a unique structural profile (Table 4.1). Typically, HA has standard lattice parameters with 'a' approximately 9.42 Å and 'c' around 6.88 Å. However, reducing these dimensions in the CF suggests a more compact crystal structure. The smaller crystallite size can be particularly advantageous for biological applications. it often increases surface area, enhancing cell adhesion and proliferation [61]. This is crucial in applications like bone grafting and dental implants, where integrating the material with the biological tissue is essential for success. in addition, HA's low degree of crystallinity is thought to resemble the structure of real bone [62] more closely. The decreased crystallinity of natural bone minerals is thought to be more compatible with the biological environment, resulting in increased bioactivity and resorption. in bone regeneration applications, this is crucial since the material will need to integrate and be replaced by native bone tissue progressively. XRD

examination of the AB and GB samples shows that it is a biphasic CaP combination of HA and β-TCP, with HA contributing 57% and 60% of the AB and GB samples, respectively, and β-TCP making up the remaining 43% and 40% of the AB and GB samples, respectively (**Table 4.1**). Critical characteristics that determine the material's biological performance, such as lattice parameters, crystallite size, and degree of crystallinity, are all reduced in the XRD patterns of AB and GB materials (**Table 4.1**). it's important to note that β-TCP is included as a secondary phase since it speeds up the resorption time and improves the bioactivity of the composite [63]. The slower resorbing HA is complemented by the more resorbable β-TCP, creating a well-balanced scaffold that encourages bone ingrowth while being eventually replaced by natural bone [64]. Consequently, the unique combination of HA and β-TCP, their specific proportions, and altered microstructural characteristics revealed by XRD make the AB and GB samples promising candidates for applications in bone tissue engineering.

Table 4.1. Lattice parameters, cell volume, and degree of crystallinity of the studied samples.

	Lattice parameters					**Phase (%)**	
Sample ID	**a(Å)**	**c(Å)**	**CV(Å)^3**	**Crystinallity (%)**	**Crystallite size (nm)**	**HA**	**Btcp**
JCDPS (HA)	9.418	6.884	528.80	----	----	100	0
HA	9.412	6.865	526.67	88.11	70.43	100	0
JCDPS (βTCP)	10.412	37.318	3503.6	——	——	0	100
β-TCP	10.080	37.420	3369.6	92.02	43.25	0	100
CF	9.025	6.798	479.59	71.03	16.46	100	0
AB	9.251	6.593	488.75	54.74	26.65	57	43
GB	8.619	6.382	410.62	52.94	25.45	60	40

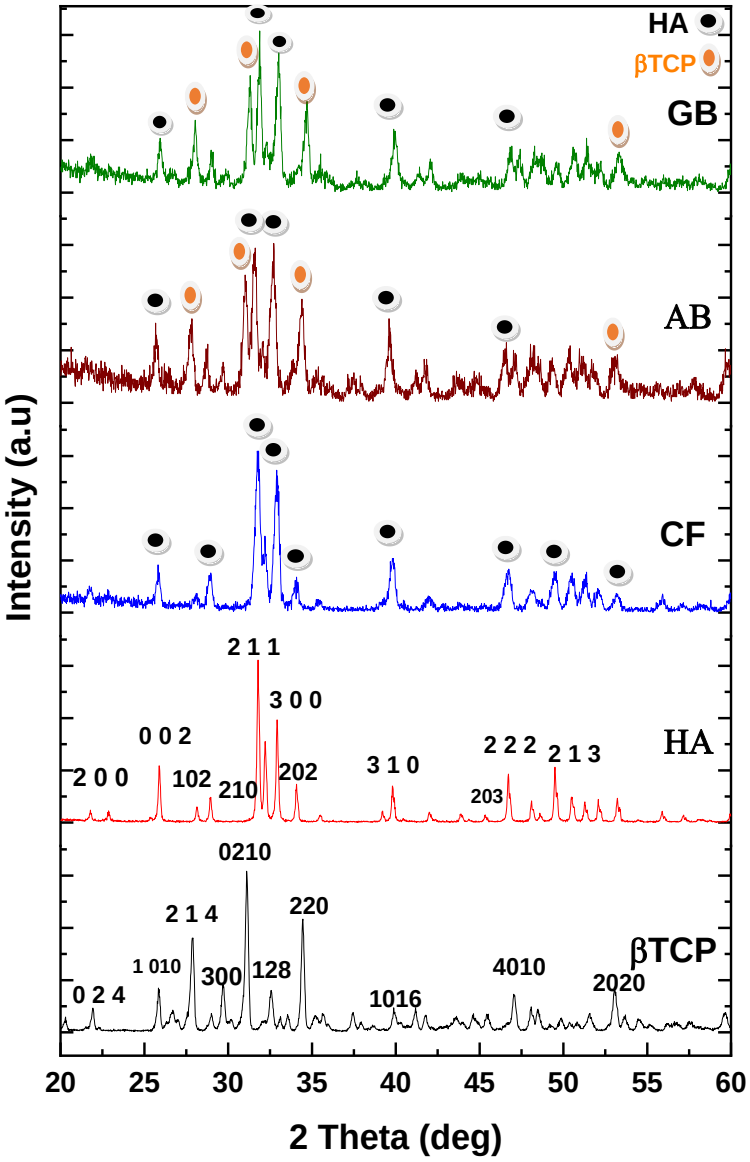

Figure 4.1. The XRD pattern of synthetic and extracted materials was calcined at 1000°C for 2h.

Figure 4.2. presents the FT-IR analysis used to identify the functional groups in the synthetic and extracted materials calcined at 1000 °C for 2 h. The FTIR spectra strongly correlated the synthesized HA and β-TCP with the CaP-extracted samples. Notably, the fish bone samples exhibited spectra almost identical to that of HA and showed significant similarity to the β-TCP pattern. This similarity confirms the presence of biphasic (HA and β-TCP) in the samples. A slight variation in peak intensities was observed in certain positions, along with the absence or faint peak at the 630 cm^{-1} position in the GB spectrum. The dominant bands at 958, 1020, and 1085

cm^{-1}, along with peaks at 565 and 601 cm^{-1}, align with the fundamental vibration modes of the PO_4^{3-} group in the HA structure. The stretching and bending modes of the hydroxyl (OH) group in HA were observed at 630 and 3520 cm^{-1}. Variations in the intensities of these OH stretching bands are attributed to the different water absorption levels on the diverse porous surfaces of HA nanoparticles. Peaks around 1640 cm^{-1} are associated with the bending mode of H_2O. The FTIR spectrum data for three types of fish powders, as presented in **Table 4.2**, exhibit characteristic peaks indicative of various functional groups. These peaks correspond to different vibrational modes of the molecular bonds within the samples.

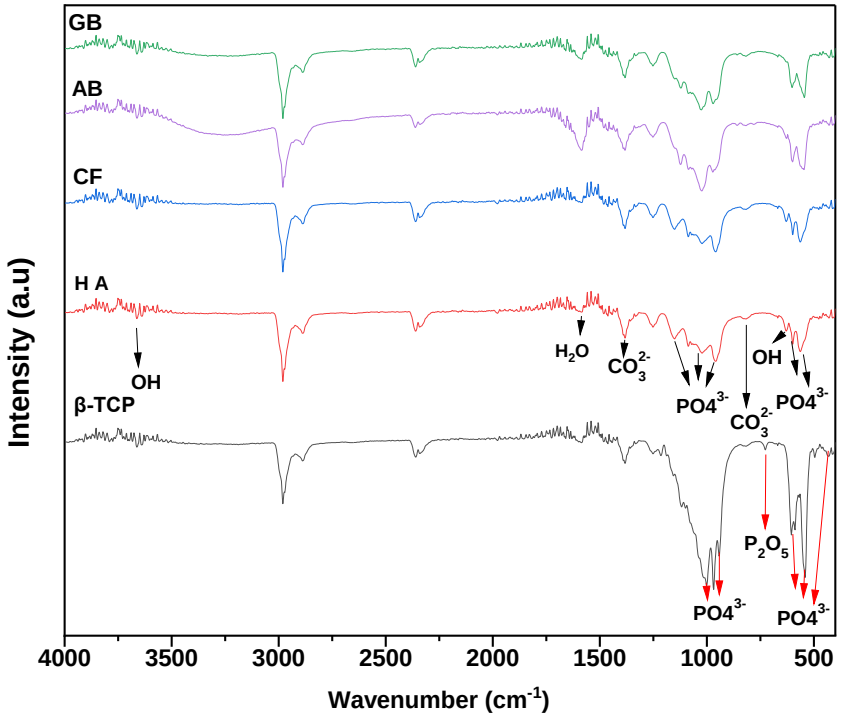

Figure 4.2. FTIR Patterns of synthetic and extracted powders sintered at 1000 °C for 2h.

Table 4.2. Characteristic peaks of synthetic and extracted materials.

FTIR Peaks (cm^{-1})					
β-TCP	**HA**	**CF**	**AB**	**GB**	**Band assignment**
547	563	563	541	543	P-O-H Stretching mode of $PO4_3$
600	599	598	602	603	P-O-H Stretching mode of $PO4_3$
_____	526	526	524	524	O-H bending mode
727	_____	_____	_____	_____	P-O bending mode of $PO4_3$
_____	815	815	817	821	C-O bending mode of $PO4_3$
965	962	962	972	975	P-O Stretching mode of $PO4_3$
1593	1606	1605	1583	1587	H-O-H bending mode
2980	2981	2977	2979	2979	C-H stretching mode
3667	3664	3664	3666	3666	O-H stretching mode

The microstructural characteristics and particle sizes of synthetic and fish bone extracted materials are depicted in Figure 4.3.(a-e). The HA sample shown in Figure 4.3.(a) has an average particle size of 105.27 nm, displaying a variety of shapes, including plate-like and spherical, attributed to the precipitation method of synthesis as reported by Antonia Ressler et al [65]. Meanwhile, the β-TCP microstructure in Figure 4.3.(b) is characterized by dense crystallites without visible gaps or fissures, averaging around 300 nm in diameter. This reflects the results obtained by Puroniene et al. [66], who synthesized β-TCP using the wet precipitation method followed by calcination at temperatures around 900°C, which is comparable to the 1000°C used in the current study, yielding particle sizes ranging from 100 to 300 nm. The fish bone sample (CF) depicted in Figure 4.3.(c) shows well-dispersed spheroid aggregates, which likely formed due to high-temperature treatments. image (GB) reveals large (Figure 4.3.(d)), clear spherical particles averaging 595 nm in diameter, while image (AB) in Figure 4.3.(e) presents irregular and agglomerated particles. The observed discrepancy between particle sizes estimated from XRD data and those obtained from microscopic images suggests particle aggregation. Additionally, HA and β-TCP were the only phases detected in all samples. The hexagonal structure of the HA phase was confirmed using unit cell software, with lattice data aligning with known crystallographic information for both natural and synthetic stoichiometric HA synthesized using the described methodology. For the β-TCP phase, the refined lattice data corroborates the composition's validity, and the results are consistent with recognized standard phase values. The variance in size, shape, and crystallinity across

these images suggests different nucleation and growth conditions during the synthesis of these HA particles. These characteristics are crucial as they determine the suitability of the nanoparticles for specific applications in regenerative medicine and tissue engineering.

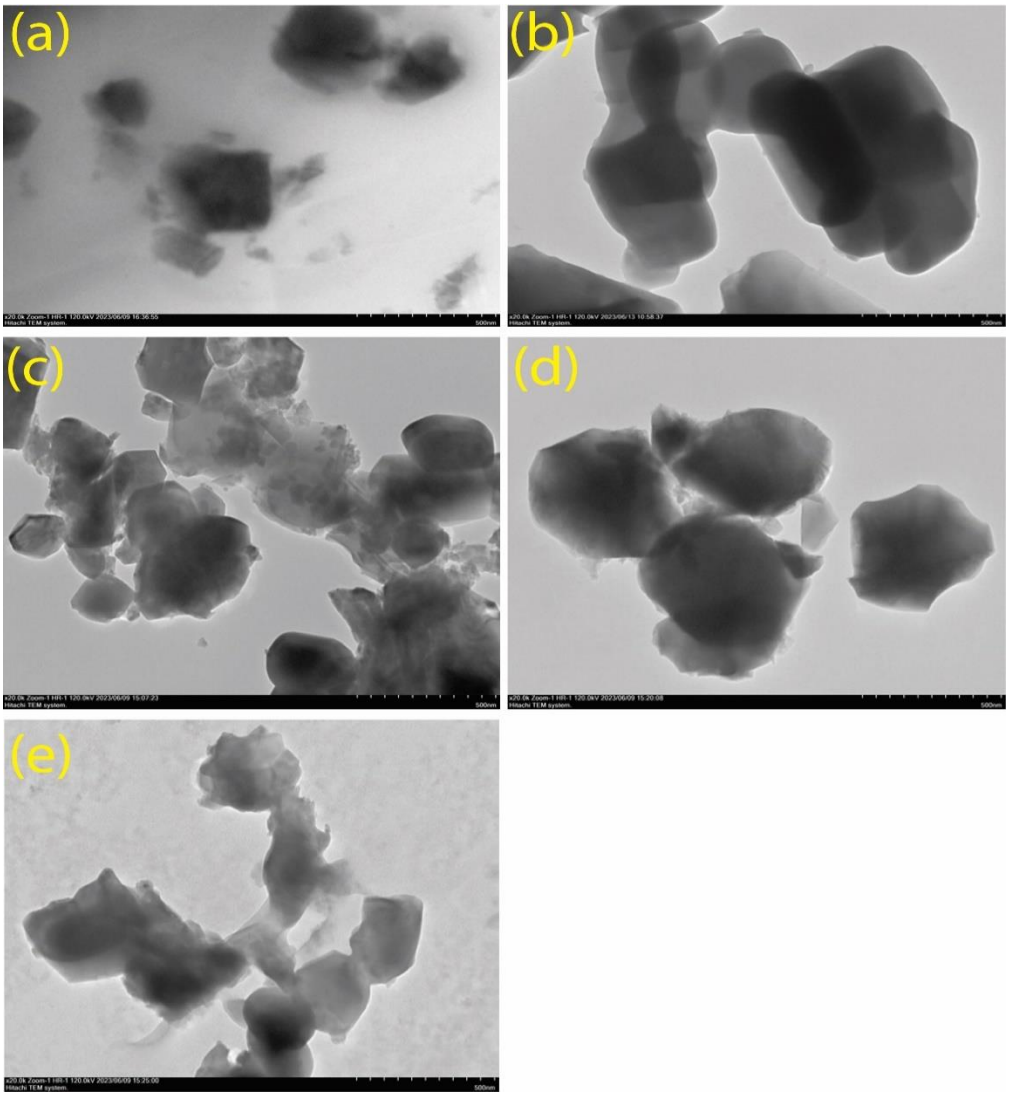

Figure 4.3. TEM images of (a) HA, (b) β-TCP, (c) CF, (d) GB, and (e) AB particles calcined at 1000°C for 2 h.

Quantitative surface analysis and compositional determination of samples can be effectively conducted using XPS technology. Figure 4.4. presents the results of an XPS analysis examining the chemical compositions of HA, β-TCP, CF, AB, and GB samples. The XPS spectra confirm the presence of elements such as oxygen (O 1s), calcium (Ca 2s and Ca 2p), carbon (C 1s), and phosphorus (P 2s and P 2p), with binding

energies of 532, 438, 348, 285, 190, and 132 eV respectively. These elements are fundamental constituents of CaP materials, and their binding energies closely match those expected for a pure phase. The binding energies for Ca 2p and P 2p peak at 348 eV and 132 eV, respectively, align with typical CaP values. The C 1s peak at 285.0 eV indicates carbonaceous impurities adsorbed on the surface [67], its present confirmed by FTIR (Figure 4.2) . The deconvoluted O 1s spectra displays two distinct peaks; one at 530.3 eV associated with oxide ions (O^{2-}), and another at 531.8 eV related to hydroxide ions (OH^-). Additionally, the binding energy of 133.6 eV from the P 2p spectrum is consistent with other compounds containing the PO_4^{3-} group. in the fish (CF) sample's graph, a subtle peak at a binding energy of 305 eV may suggest the presence of sodium (Na). The range between 0 to 200 eV features a series of smaller peaks that could correspond to elements such as phosphorus (P 2s), silicon (Si 2s), sodium (Na 2s), calcium (Ca 3s), and oxygen (O 2s), as suggested by the study conducted by N. Ohtsu et al. [68]. These peaks reinforce the complex nature of the surface chemistry of the fish bone-derived samples.

The Ca/P ratio is significant, particularly in the field of biomaterials, as it is one of the factors that can influence the bioactivity and biocompatibility of materials, especially those intended for bone substitution or repair. The Ca/P ratios given in Table 4.4. suggest varying degrees of similarity to natural bone mineral, which is typically reflected in the ratio found in HA, around 1.67. The β-TCP sample has the lowest Ca/P ratio of 1.32, which is characteristic of this material. Although it doesn't match the Ca/P ratio of natural bone, β-TCP is valued for its resorption rate and is widely used in bone grafting where a faster material turnover is beneficial. The CF sample has the highest Ca/P ratio (1.67), matching the ratio of natural HA. Since CF is pure HA, this indicates that it is very close to the mineral composition of natural bone, potentially making it highly suitable for applications that require materials with characteristics similar to natural bone, such as implants or scaffolds for bone regeneration. AB and GB samples, being combinations of β-TCP and HA, show Ca/P ratios of 1.51 and 1.57, respectively. These ratios are lower than that of pure HA, which can be attributed to the presence of β-TCP. β-TCP has a lower Ca/P ratio, and its inclusion would reduce the overall ratio in a composite material. Despite the lower ratios, these composites can have their own advantages, such as controlled resorbability due to the presence of

β-TCP, which can be beneficial for certain applications where gradual replacement of the material by natural bone tissue is desired.

Table 4.3. The atomic ratio of elements present in both the synthetic and extracted materials.

Sample id	Atomic (%)								Ca/P Ratio
	Ca 2p	P 2p	O 1s	Na 1s	Mg 2s	C 1s	Si 2p	S 2p	
β-TCP	17.096	12.944	43.503			26.456			1.32
HA	21.088	12.824	49.798			16.290			1.64
CF	17.162	10.224	53.533	2.140	4.089	11.613		1.241	1.67
AB	20.261	13.390	54.273	0.669	1.033	10.375			1.51
GB	19.003	12.035	49.552	0.658	2.062	16.293	0.397		1.57

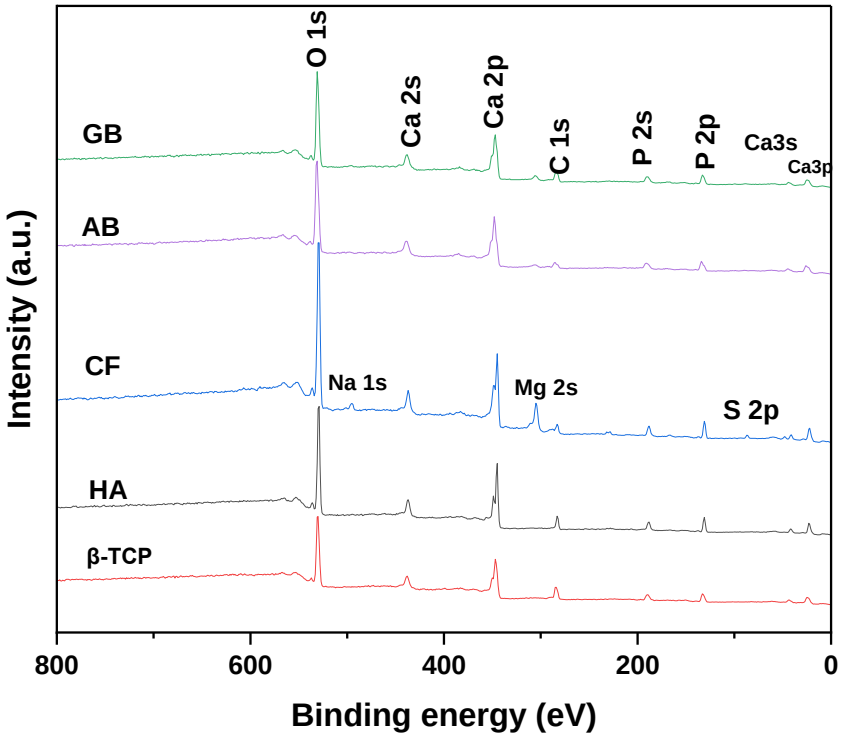

Figure 4.4. XPS patterns of synthetic and extracted materials.

4.2. IN VITRO DEGRADATION ANALYSIS

The in vitro degradation behavior and pH values of various samples incubated in PBS at 37°C for 14 days are depicted in Figures 4.5.(a-c). Figure 4.5.(a) demonstrates the multi-staged release of Ca^{2+} ions into the PBS. initially, from day 0 to 1, all materials began to degrade, leading to an uptick in Ca^{2+} concentration, with β-TCP showing the

most significant release. With a prolonged immersion period to 3 days, variations in Ca^{2+} concentration were observed; HA and CF saw an increase, whereas β-TCP, GB, and AB experienced a decrease. This could be due to the precipitation of [P] and [Ca] ions forming an apatite layer and thus reducing their concentration in the solution [69]. The third stage of immersion, from days 3 to 7, significant a decrease in the releasing of Ca ions for β-TCP, a slight rise for GB, a more substantial increase for CF and HA, and a pronounced surge for AB. Finally, in the last stage from days 7 to 14, all groups showed increased Ca^{2+} release, with HA, CF, and AB experiencing the highest release rates. This suggests the formation of an apatite layer due to enhanced interactions with the PBS [69].

Figure 4.5. (b) illustrates the P^{5+} release profile, indicating an immediate release in the PBS, which was especially pronounced for GB. Subsequent stages showed a decrease in P^{5+} ions for β-TCP and GB, stability for AB, and slight reductions for CF and HA, possibly due to apatite layer formation [69]. in the third phase, a notable rise in P^{5+} release occurred for β-TCP and AB, with moderate increases for CF and GB, while HA saw continued increments. The final stage exhibited the maximum P^{5+} release for β-TCP, AB, and CF, with modest increases for HA and GB.

The pH variations of the PBS solution during sample immersion, presented in Figure 4.5.(c), initially increased in the first 24 hours due to ionic exchange processes, followed by a gradual decrease in pH for CF, a slight reduction for HA, β-TCP, and GB, and then stabilization for AB and HA after three days. From days 7 to 14, a consistent decrease in pH was noted. The PBS pH tended to decrease with prolonged immersion time, indicating ongoing sample degradation in the solution.

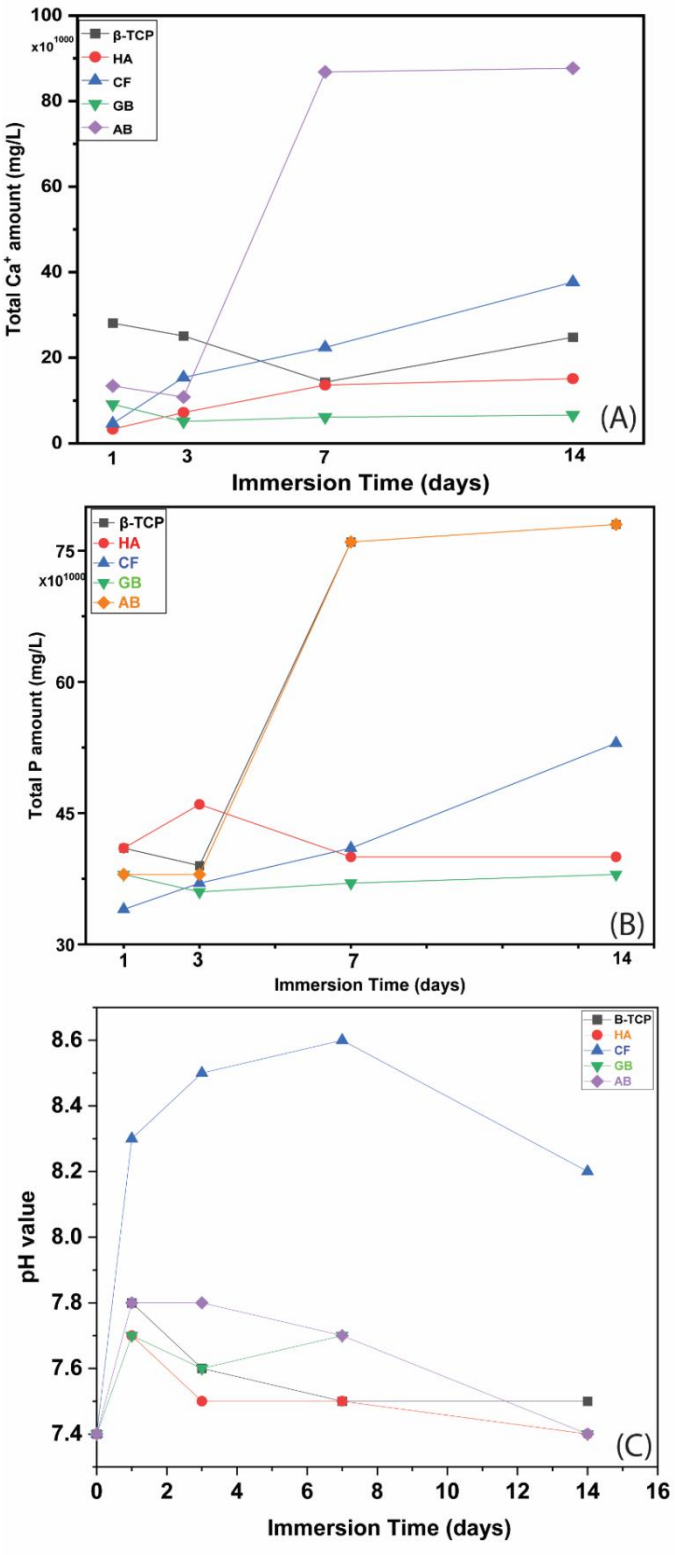

Figure 4.5. Releasing profile of (a) Ca ions and (b) P ions. (c) pH value vs immersion time.

PART 5

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORKS

In conclusion, this comprehensive study successfully synthesized and characterized synthetic and extracted CaP materials, demonstrating their potential for biomedical applications, particularly in bone tissue engineering. The use of advanced techniques such as XRD, FTIR, XPS, and TEM provided in-depth insights into their microstructural and compositional characteristics. The findings revealed that these synthesized materials closely resemble natural bone regarding crystallinity and Ca/P ratio, making them highly suitable for applications like bone grafting and implants. in vitro degradation tests conducted on the CaP materials showcased effective degradation rates for all the samples. Additionally, the accelerated interaction between the CaP samples and the PBS solution led to the formation of an apatite layer. Significantly, the CaP extracted from AB fish bones exhibits considerable potential for use in bone substitute materials. The compositional and structural attributes of these materials, particularly their close resemblance to biological CaP, render them ideal for biomedical applications. Their higher degradation rates suggest an enhanced capability to integrate into the body, promoting bone regeneration and repair. This finding paves the way for promising future research and development in bone tissue engineering and orthopedic treatments. Such materials could offer a sustainable and effective alternative to conventional bone grafts in these fields.

Future research on CaP materials derived from fish bones for bone tissue engineering could focus on a series of in vitro and in vivo studies. These should include biocompatibility assessments through cell culture experiments, in vivo bone regeneration models using animal subjects, and comparative analyses against commercial biomaterials. Long-term degradation, resorption rates, and mechanical strength studies are crucial to validate their efficacy and safety. Eventually, progressing towards human clinical trials will be essential to establish their practical applicability in orthopedic treatments, offering a sustainable alternative to traditional bone grafts.

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RESUME

Lemana CHEIKH was graduated from primary education from El Wefa School in Arafat and middle and high school from El Islah in 2017. He graduated from the University of Nouakchott El Asriya, Faculty of Technical Sciences in Nouakchott 2020, He started the Master's Program at Karabuk University, Department of Biomedical Engineering in 2021.