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Development of Calcium Phosphates from Waste Resources and Fabrication of Calcium Phosphate-Chitosan Composites

By

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***I dedicate this thesis to my beloved parents,
husband, son and daughter***

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ABSTRACT

Biowastes from agriculture, sewage, household wastes and industries comprise promising resources to produce biomaterials whilst reducing environmental impact. This study focused on utilising waste-derived materials (i.e., eggshells as calcium source, struvite as phosphate source and CH_3COOH as dissolution media) to produce value-added products (i.e., calcium phosphate (CaP) derived biomaterials) using three different routes such as continuous flow hydrothermal synthesis, ball milling followed by sintering and flame spheroidisation. Fabrication of composites (scaffolds and films) using commercial chitosan (Ch) and CaPs prepared using eggshells and struvite via three different methods mentioned above was also explored. The prepared materials were characterised via XRD, TGA/DSC, FEG-SEM, EDX, FTIR and TEM analysis.

Nanocrystalline hydroxyapatite (HA) formation was observed by reacting eggshell solution (prepared using HNO_3) and diammonium hydrogen phosphate solution whereas magnesium whitlockite (Mg-WH - the second most abundant bone mineral), and HA were produced as a single phase or biphasic CaPs by reacting struvite with either calcium nitrate tetrahydrate or eggshell solution at 200 °C and 350 °C via continuous flow hydrothermal synthesis, where waste-derived precursors solutions were prepared using either HNO_3 or CH_3COOH . Structural characterisation and compositional analysis confirmed the sheet-like morphology of nano HA formation (with crystallite size ranges between 29 - 81 nm) at 200 °C. However, HA tubes (32-212 nm inner diameter and 15-49 nm wall thickness) and rod morphologies (32 – 86 nm) were observed at 350 °C using NH_4OH and NaOH as pH buffer, respectively. Rhombohedral-shaped Mg-WH (23 – 720 nm) along with tube (50 – 290 nm in diameter with 20 – 71 nm wall thickness) and/or elliptical morphologies of HA (273 – 522 nm in width) were observed at 350 °C when using both dissolution media (HNO_3 and CH_3COOH) to prepare the eggshell and struvite solutions where NH_4OH was used as the pH buffer. The Ca/P (atomic%) ratio of the samples produced ranged between 1.3 and 1.7, indicating formation of non-stoichiometric HA and Mg-WH.

A single phase HA along with brucite [$\text{Mg}(\text{OH})_2$] was formed by simple ball milling of calcined eggshell powder and struvite, while biphasic CaPs (i.e. HA and Mg-WH) alongside MgO was

produced at higher sintering temperatures (i.e. 900 and 1150 °C) using either raw or calcined eggshells and struvite. The ranges of crystallite size of HA and Mg-WH were found to be 30.5 – 80.6 and 28.45 – 82.5 nm, respectively.

The CaPs microspheres were manufactured successfully from ball-milled raw/calcined eggshell-struvite of two granules size ranges (63 – 100 and 100 – 160 μm) via flame spheroidisation process. Single-phase of α -TCP was formed for the ball milled raw eggshell - struvite samples (smaller size granules), whereas biphasic CaPs (i.e. α -TCP and HA) was formed when calcined eggshell was used with struvite for ball milling. The Ca/P ratio of microspheres were found to be within the range of 1.5 – 1.65.

CaP-Ch composites (i.e. scaffolds and film) were fabricated successfully via a freeze drying and casting method. Resonance acoustic mixing (RAM), a newly developed mixing method, was used to prepare a homogeneous composite suspension prior to freeze drying. Crosslinking of the scaffolds was also performed by using genipin, a naturally-derived crosslinker. Morphological, structural, compositional, swelling properties, mass loss and mechanical properties were evaluated for the composites. Interconnected porous structure was observed for all the scaffolds. However, the reduction in porous structure and mass loss and no significant change in mechanical properties for the cross-linked scaffolds compared to bare scaffolds was observed. This was due to the partial crosslinking of scaffolds by genipin, confirmed via FTIR. Films of the same formulations (except using CaPs derived via hydrothermal synthesis) were also fabricated using casting method and their properties were evaluated. The films containing commercial HA exhibited highly interconnected porous structure. However, a film comprising ball-milled CaPs showed a moderate range of swelling percentage (211 %) with highest mass loss (about 34 % after 96 hrs) in comparison to other formulations.

This study shows that use of eggshells and struvite offer a promising resource as potential sustainable precursors, along with acetic acid as sustainable dissolution media to produce calcium phosphates with varying morphologies, microspheres and their composites with chitosan for biomedical applications.

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COVID19 IMPACT STATEMENT

I had passed a very stressful (physically and mentally) life during Covid period. As my research work was laboratory-based, it was really hard to progress without any laboratory access or limited access. The uncertainty of reopening the university and getting back to normal life, was very frustrating. Moreover, I got infected with Covid and I suffered a lot, especially with breathing difficulties. I became very weak and got tired very quickly. Till now, I am suffering from breathing difficulties sometimes and cannot do work like before.

In response to an extraordinarily difficult year, I faced significant challenges in my research work. I was 2nd year Ph.D. student at that time. During the first lockdown, all experimental work was suspended. And, during the second and third lockdowns, all the research group members had to work in the laboratory in a rota basis to maintain a safe distance.

My research work was mainly based on hydrothermal synthesis. I had to prepare solutions in one lab whereas, synthesis was conducted in another lab of a different building. One lab mate had to be present during the experiment to run the equipment. Solution preparation was continuous and required a longer time (3-4 days) and freshly prepared solutions were recommended for the synthesis process to get accurate results. Because of the limited access to the lab along with the rota system, my research work especially the hydrothermal synthesis part was affected significantly and did not progress as expected. Moreover, due to the limited access to the use of the instruments, a longer time was needed to complete the characterisation which also affect the quality of the developed product to be characterised. More importantly, during the restricted period, I was not allowed to operate instruments myself, for which I had to wait for a long time most of the time (especially for highly demanded instruments like XRD, FEG-SEM) to get the results of my samples. Also, when we were allowed to use instruments ourselves, we had to go through a long documentation process and had to book the highly demand instruments several weeks before, even more than a month before. All these restrictions during the Covid period limited the quality of my research work which was very disappointing.

I had tried to manage my project during this difficulty time by reading literature and writing and analysing the previous experimental data. My supervisor monitored the progress of my work and guided regularly and provided mental support all the time in research group meetings via Microsoft teams.

LIST OF PUBLICATIONS

Journal article

1. Md Towhidul Islam, Nur Aisyah Nuzulia, Laura Macri-Pellizzeri, Farah Nigar, Yessie W. Sari and Ifty Ahmed, Evolution of Silicate Bioglass Particles as Porous Microspheres with a View Towards Orthobiologics. ***Journal of Biomaterials Applications***, 2022, 36(8): p. 1427-1443
2. Farah Nigar, Amy-Louise Johnston, Jacob Smith, William Oakley, Md Towhidul Islam, Reda Felfel, David Grant, Edward Lester, and Ifty Ahmed, Production of Nano Hydroxyapatite and Mg-Whitlockite from Biowaste-Derived Products via Continuous Flow Hydrothermal Synthesis: A Step towards Circular Economy. ***Materials***, 2023, 16(6): 2138.

Conferences

Farah Nigar, Amy-Louise Johnston, Ammar Alatta, Jacob Smith, William Oakley, Md Towhidul Islam, Reda Felfel, David Grant, Edward Lester and Ifty Ahmed. Biowaste-Derived Nanocrystalline Hydroxyapatite and Mg-Whitlockite via Continuous Flow Hydrothermal Synthesis. 7th ISHA 2021 International Conference (Virtual Event), 26th – 28th October, 2021 (Oral).

ABBREVIATIONS

ACP	Amorphous Calcium Phosphate
CaPs	Calcium phosphates
CDHA	Calcium-deficient hydroxyapatite
<i>c.a.</i>	Circa
Ch	Chitosan
3D	Three dimensional
DCPA	Dicalcium phosphate anhydrous
DCPD	Dicalcium phosphate dihydrate
DSC	Differential scanning calorimetry
EDX	Energy dispersive x-rays
FAP	Fluorapatite
FTIR	Fourier transform infrared
HA	Hydroxyapatite
HCA	Hydroxycarbonate apatite
ICPMS	Inductively coupled plasma mass spectroscopy
MCPA	Monocalcium phosphate (anhydrous)
MCPM	Monocalcium phosphate monohydrate
Mg-WH	Mg-Whitlockite
nHA	nano-hydroxyapatite
OCP	Octa calcium phosphate
PBS	Phosphate buffered saline
SEM	Scanning electron microscope
TEM	Transmission electron microscopy
TGA	Thermogravimetric Analysis
TTCP	Tetra calcium phosphate
XRD	X-Ray Diffraction
α -TCP	α -tricalcium phosphate
β -TCP	β -tricalcium phosphate
ρ	Density

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Chapter 1

INTRODUCTION

1.1 Overview

Developing sustainable materials and moving towards a circular economy is currently a hot topic for all major industries across the world. The use of waste materials as a valuable resource of renewable raw materials could be utilised to address this issue [1]. Over the last few decades, scientists are investigating some bio-wastes, like bovine bones, fish bones, avian eggshells, cuttlefish shells, oyster shells and corals [2, 3] to develop biocompatible materials [4-6]. Having high-quality calcium including some natural trace elements like K, Na, Mg, Si and Sr, eggshells are emerging as a potential source of calcium (Ca) for the production of hydroxyapatite (HA) and other calcium phosphates (CaPs) and thus have a promising future if reused [7]. On the other hand, phosphorous (P) recovery from P-rich wastewater [8-10] through struvite formation [11] could be more effective for both environmental protection and nutrient recycling [12]. Struvite contains 39% phosphate [13] and thus has great potential to use as an alternative P source [14]. In addition, production of acetic acid from food waste and its utilisation is another approach towards the sustainability and circular economy.

In the era of advanced science, nanotechnology has become an increasingly important for developing nanomaterials to assist in overcoming many challenges and limitations in the use of biomaterials in tissue engineering [15]. For example, having greater surface area than coarse crystal, nano hydroxyapatite (nHA) exhibits improved densification which enhances its mechanical properties including fracture toughness [16, 17]. Moreover, nHA as an implant material has shown better bioactivity and improved biocompatibility over coarser HA based implants [18, 19].

Several methods, such as in situ biomimetic process [20], hydrothermal method [21], sol-gel precipitation technique [22], ultrasonic atomisation [23], ultrasonic coupled sol-gel synthesis [24], a wet ball-milling method followed by sintering [25] have been evolved for synthesising nano-crystalline HA as well as mixed phases of CaPs [26]. Among them, continuous-flow hydrothermal synthesis appeared as a resourceful method that could meet the growing

demand for industrially sustainable routes to produce nanomaterials with different morphologies.

Researchers are nowadays, focusing more on reducing the usage of harmful chemicals and implementing green approaches from renewable, sustainable resources as starting materials [27]. Ball milling is considered as a green technology with enormous potential and could be applied to a wide range of materials such as cellulose, HA, ilmenite, metal oxides, polymers, etc [28]. Moreover, this technique is cost-effective, reliable, reproducible with high yield and ease of operation [28]. Ball milling, however, produces amorphous or low crystalline products of CaPs, which may require further sintering of the samples depending on a wide range of applications. Pure HA or multiple phases of CaPs with high crystallinity could be obtained by sintering the ball-milled substances [29].

In the advanced biomedical field, microspheres have growing attention over irregular-shaped particles for use in biomedical applications such as drug delivery, bone tissue engineering and regeneration, absorption and desorption of substances etc. [30]. They can be manufactured in a uniform size and shape and possesses improved flow properties, superior packing densities and larger surface areas [31]. These properties allow sufficient therapeutic coatings, an increase in ion release and degradation rate [30]. Moreover, microspheres can be delivered to the specific target site via minimally invasive injection providing significant benefits for treatment and recovery to the patients [31].

Calcium phosphates based ceramic microspheres offer excellent biocompatibility, osteoconductivity and adequate mechanical properties in the field of orthopaedics, dentistry and the pharmaceutical sectors and thus researchers nowadays have a common interest in this category, particularly HA [32, 33], and β -tricalcium phosphate (β -TCP). Microspheres of calcium phosphate-based ceramics were mainly prepared from synthetic sources of Ca and P [33-35]. Microspheres can be prepared via different methods such as precipitation, spray pyrolysis, electrospraying, emulsification processes, flame spheroidisation, etc. Among them, the flame spheroidisation process is very fast, simple and inexpensive [30].

Bone grafting is a current clinical approach to treat critically damaged bone, which caused by a severe fracture, zero or less possibility for bone fusion, high energy trauma, development deformities, bone diseases like osteonecrosis and cancer, bone infection (osteomyelitis) etc

[36]. However, bone grafting have some limitations, such as the potential risk of infectious diseases which limits its application [37, 38]. To overcome this, a wide variety of bone graft substitute are intensively investigated. Among them scaffolds have drawn much attention [39, 40]. Scaffolds provides a 3D structure to support cell growth, migration, proliferation and differentiation [39]. In recent years, research on CaPs containing chitosan (Ch) based scaffolds for bone tissue engineering has increased significantly [39, 41, 42]. The ceramic materials assist to enhance both osteoconductivity and mechanical properties of Ch scaffolds. This type of scaffolds supports the proliferation and osteogenic differentiation of pre-osteoblasts and promotes bone recovery [37]. In order to enhance the mechanical properties as well as the stability of the scaffolds in biological environment to deliver drug over a desired period of time, crosslinking of the scaffolds has widely been conducted. Several synthetic crosslinkers such as glutaraldehyde, formaldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate are used to crosslink chitosan [43], however, they are cytotoxic and may damage the biocompatibility of a chitosan delivery system. Hence, naturally derived crosslinking agents have a growing interest recently for their low toxicity [44].

Genipin (G) is a natural crosslinker derived from the fruits of *Genipa Americana* and *Gardenia jasminoides* [44, 45] which offer lower toxicity, higher biocompatibility, comparable mechanical strength and resistance against enzymatic degradation of the materials [46]. Genipin reacts with amino groups of chitosan rapidly and promotes the strength and stability of the scaffolds [44].

Chitosan-CaPs films are biocompatible and can be applied in bone tissue engineering for the surface modification of bone implants, restoration of damaged bones, to enhance biocompatible interface between osteoblast cells and microscale inorganic materials, and to improve the osteoconductivity of bone regeneration [47].

1.2 Aims and Objectives

The aim of this project was the development of calcium phosphates (CaPs) from renewable waste resources such as eggshells and struvite as precursor materials and acetic acid (can also be prepared from food-waste) as a dissolution media with a view towards sustainable manufacture of CaP based biomaterials.

The work included the following specific objectives:

- Synthesis of CaPs via continuous flow hydrothermal synthesis process from waste-derived precursors using three different combinations: 1) Eggshell and Di-ammonium hydrogen phosphate, 2) Calcium nitrate tetra-hydrate and Struvite, and 3) Eggshells and Struvite. Exploring alternative sustainable dissolution media (i.e. acetic acid). Characterisation of developed materials via XRD, SEM, EDX, FTIR and TEM analyses.
- Exploring another sustainable route, ball milling followed by sintering and/or flame spheroidisation to prepare CaPs particles /microspheres from natural waste resources (such as eggshells and struvite). Characterisation of the materials prepared via XRD, TGA/DSC, FEG-SEM, EDX and FTIR.
- Fabrication of composites (scaffolds and films) using commercial chitosan (Ch) and CaPs with varying morphologies prepared using eggshells and struvite via three different methods (hydrothermal synthesis, ball milling followed by sintering, and ball milling followed by flame spheroidisation process). Exploring new technique (i.e. Resonance acoustic mixer) to obtain a homogeneous mixture of the composite suspension. Characterisation of the composites prepared via FEG-SEM, EDX and FTIR, swelling, mass loss and mechanical properties.

1.3 Thesis Outline

The outcomes of the aims and objectives have been divided into several chapters as outlined below:

Chapter 2: reviews the literature relating to sustainability and circular economy, materials recovery, reuse and recycling, various types of biowaste materials and their impact on environmental, ecosystems and human health. Developing affordable healthcare in Low

Middle-Income Countries (LMICs) and bone tissue engineering approaches using biowastes were also considered. This Chapter also reviews different types of bone repair materials and procedures such bone grafts, bone grafts substitutes, bioactive inorganic materials, biodegradable polymers (i.e. Chitosan), various types of CaPs and composites (scaffolds and films).

Chapter 3: Presents the materials and methods used throughout this thesis.

Chapter 4: This chapter focuses on the hydrothermal approaches employed in the development of CaPs with varying morphologies from sustainable waste resources such as eggshells as Ca and struvite as P source. The materials produced had been characterised using XRD, SEM, EDX, FTIR and TEM analyses. The effect of pH, base solution, dissolution media and temperature on the prepared samples were also investigated.

Chapter 5: This Chapter focuses to explore another sustainable route ball milling followed by sintering and/or flame spheroidization to prepare CaPs particles /microspheres from natural waste resources (such as eggshells and struvite) for biomedical applications, where no harmful chemicals were used. The precursor materials were first milled in a planetary ball mill and then ball-milled samples were sintered at 700, 900 and 1150 °C or processed via flame spheroidisation and their characterisation was conducted via XRD, TGA/DSC, FEG-SEM, EDX and FTIR.

Chapter 6: This Chapter focuses on the fabrication of composites in the form of scaffolds and films via freeze drying and casting methods, respectively using commercial chitosan (Ch) and CaPs with varying morphologies prepared using eggshells and struvite via three different methods (hydrothermal synthesis, ball milling followed by sintering, and ball milling followed by flame spheroidisation process). Resonance acoustic mixer was used to get a homogeneous mixture of the composite suspension. Characterisation of the scaffolds and films were conducted via FEG-SEM, EDX and FTIR and the swelling properties, mass loss and mechanical properties were also evaluated.

Chapter 7: Conclusions of the findings of all the works carried out in this thesis including the suggestions for the scope of future works.

Chapter 2

LITERATURE REVIEW

2.1 Sustainability and Circular Economy

Sustainability means to avoid the reduction of natural resources to maintain an ecological balance. To ensure a green environment sustainability is attracting great attention worldwide [48]. Irresponsible disposal of waste materials, generated from different farming processes such as crop production, rearing of livestock or fish farming, create an adverse effect on the environment. Animal manure, agricultural by-products like bagasse, rice husk, crop residues and wastes from poultry or slaughter houses are solid bio-wastes that contaminate soil and water resources after their landfilling. Some wastes including non-pretreated eggshells, fish wastes etc. also cause odour pollution. In addition, inappropriate management of fish farms and industry wastes are harmful for the environment of marine areas. As a consequence, wastes disturb the overall ecosystem by causing detrimental effect on human, plant, animal and aqua life [49]. Therefore, bio-waste management has become obligatory and a crucial strategy in global waste management nowadays. Research to find an effective way for the proper utilisation of bio-wastes will assist to protect the environment as well as global health quality [50]. Controlled recycling and reusing of wastes towards the production of value-added products is the key to achieve the sustainable development. Consumption of bio-waste as a biomaterial for medical surgery and therapeutics proved great value to these types of wastes [51]. Some wastes contain active compounds that have value in medical applications. Researches are currently focused on the potential of bio-wastes to use as effective chemical feedstock and as renewable raw materials [48]. Several studies have already provided that many bio-wastes can be effectively served as an initial material to prepare biocompatible materials [4-6]. Bovine bones, fish bones, avian eggshells, cuttle fish shells, oyster shells, and corals are some bio-wastes which are commonly used as a feedstock in biomedical research [3]. They can be successfully converted into biomaterials [3, 22]. Among them, eggshells have received much attention to the researchers. It can be potentially used in medical and dental therapy. Moreover, except eggshells, the continuous use of the other resources may lead to their extinction [3].

The circular economy is one of the major challenges and interesting opportunities for today's world. The circular economy means to keep resources in productive use at their highest value, for as long as possible. The present trend in every sector is a linear economy which is a 'take, make, dispose' model of production [52]. But as the earth's assets are not unlimited, we need to consider all materials as precious resources and should not throw anything away. Reuse is the most economically and environmentally favourable strategy within a circular economy as well as sustainable development [53] .

A circular economy aims to eliminate 'waste', by prolonged product life and/or 'looping' the product or its constituent materials back into the system to be reused. Recovery of any material makes them not to be obsolete, reinstate their value, and thus return them to the economic system [54]. By practicing circular economy, wastes can be transformed into valuable raw materials which results in better resource productivity. This concept helps to reduce wastes as well as lessen the environmental impacts of the production and consumption globally. Novel materials can be reused, recovered and recycled with the help of taking right strategies and methods. Thus, circular economy provides a more competitive UK economy. However, there are also some problems with developing a circular economy. Recovering value from wastes involves complex processes. Likewise, during the development method of a new material, new waste management infrastructure should be designed from the beginning so that it can be used further retaining its economic and environmental value [52].

2.2 Bio-waste materials

2.2.1 Definition of bio-waste

Generally, bio-waste is the organic waste derived from natural plant or animal resources. According to the proposal of The European Commission, bio-waste includes biodegradable waste from garden and parks, food and kitchen waste from households, restaurants, caterers and retail premises, and food processing plants [55]. However, Xu *et al.* [56] reported that the literature expands the group of bio-waste on a more general basis to domestic and sewage wastes, manure, food wastes and residues from forestry, agriculture and fisheries [57-59].

Bio-waste contains a huge amount of organic and inorganic matter. As anticipated by The World Bank, bio-waste from municipal solid wastes of the urban areas worldwide may reach 2.2 billion tonnes per annum by 2025 and waste generation rates might double over the next two decades in developing countries [56].

2.2.2 Environmental impacts of bio-waste materials

2.2.2.1 Air pollution, climate change, soil and water contamination

After the disposal of food and green waste in a landfill, it decomposes in an anaerobic environment producing greenhouse gases (GHG), i.e, carbon dioxide (CO₂) and methane (CH₄) and nitrous oxide (N₂O). CH₄ can absorb heat in the atmosphere significantly and thus causes dreadful effect on climate in short period of time. Therefore, focusing on the reduction of food and green waste has the greatest impact of any other solid waste component from a climate perspective. After its release to the atmosphere, CH₄ is 84 times effective at entrapping heat in the atmosphere than CO₂ in the first two decades [60, 61]. CO₂ is another dominant GHG, which is released including air pollutants and particulate matter to the atmosphere by the transportation and treatment of wastes after collection [61]. Inadequate waste management not only promotes climate change and air pollution, but also might contaminate soil and water which directly impacts on many ecosystems and species [61]. Landfills are the third largest anthropogenic source of CH₄, a particularly potent greenhouse gas accounting for approximately 11% of estimated global CH₄ emissions, and almost 800 Mt CO₂e [62]. Currently the main environmental threat from bio-waste (and other biodegradable waste) is the production of CH₄ from such waste decomposing in landfills, which accounted for some 3% of total greenhouse gas emissions in the EU-15 in 1995. Nordahl *et al.* [63] studied a comprehensive life-cycle assessment for organic municipal solid waste which consist of landfilling, composting, dry anaerobic digestion (AD) for the production of renewable natural gas (RNG) and electricity using reported and measured data. They reported that landfilling emitted the highest amount of GHG which is 400 kg CO₂e per tonne of organic waste.

2.2.2.2 Effects on ecosystems and human health

Some ecosystems, like the marine and coastal ones, can be severely affected by poor management of waste, or by littering. Marine litter is a growing concern, and not only for aesthetic reasons: entanglement and ingestion constitute severe threats to many marine species [62].

The contamination in freshwater sources and soils allows fish and crops to consume toxic chemicals which subsequently ends up to our dinner tables [62]. Solid waste management affects everyone; especially those who are seriously ill may lose their lives. People may lose homes from landslides of waste dumps. Working in unsafe waste-picking conditions also affects people's health [64].

2.2.2.3 Indirect impact on environment

Indirect effect of biowaste involves a loss of raw materials and other inputs utilised during the production, transport and consumption phases of any product, if proper recycling or recovering from waste is not carried out [62].

2.2.3 Materials Recovery, Reuse and Recycling

In recent years, the potential of biowaste has received growing attention by academic and industrial communities because of their chemical richness along with the enormous production and widespread existence. The conversion of biowaste to value added products is one of the most promising perspectives for an effective reduction of the environmental impact of biowaste as well as economic profit [56]

The most common disposal methods of biowastes are microbial decomposition under either aerobic or anaerobic conditions, incineration and landfilling [56]. Incineration is a recovery process of energy from waste which can be used to produce heat or electricity as an alternative/renewable source that replace coal or other fuels. Energy recovery of waste can thus help to reduce GHG emissions. Recycling is a more effective tool for the significant reduction of GHG and other emissions [62]. To alleviate GHG emission, disposal and treatment operation should be improved through landfill gas recovery and improved landfill

systems, controlled composting, state of the art energy recovery systems, and separating valuable materials from the waste through improved collection system [62].

Miller *et al.* [60] mentioned about a waste hierarchy (see Figure 2.1) which assisted to evaluate the priorities of preferred programme for environmental protection along with resource and energy consumption based on sustainability. The top of the pyramid represents the prioritised actions aiming to reduce the actions at the bottom. This hierarchy is a general agreement of high-quality waste management systems by following which product's lifecycle can be managed in the most resource-efficient and environmentally sound way [60].

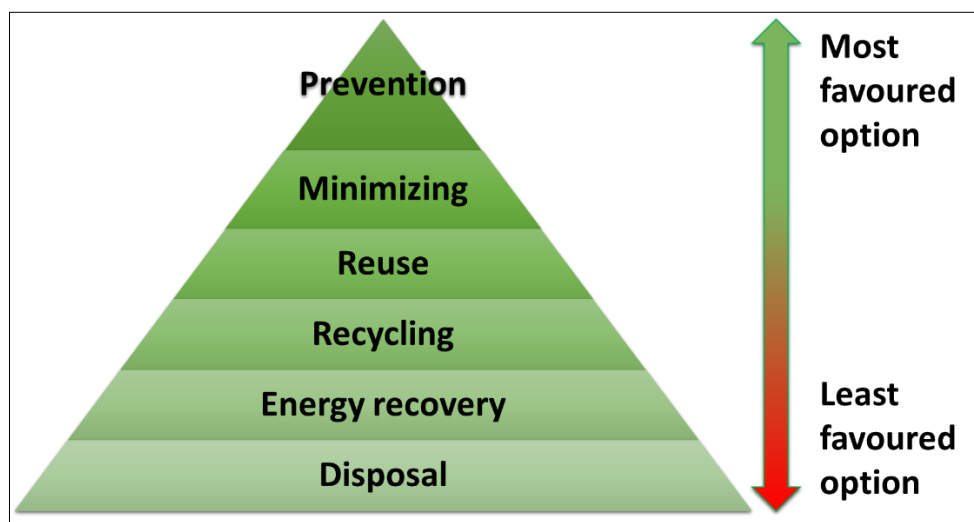


Figure 2.1: The waste management hierarchy [60].

An example of the generation of high value-added products from food wastes is production of acetic acid, which meets the demand of expansion of acetic acid production process and minimise the depletion of the world's oil resources. Hence, this type of production from food wastes is able to increase the annual yield of acetic acid while decrease the food wastes and production cost [65].

Bio-waste can be fundamentally converted into a broad range of end use products and materials either physically, chemically or biologically [56]. Xu *et al.* [56] reported on the fabrication of bio-waste-derived nanomaterials using readily available sources such as collagen, chitin and chitosan, hydroxyapatites and bio-silica/silicates, and other carbon-based feedstocks as a starting materials, which can be used in a wide variety of sectors from the

biomedical field of drug delivery and tissue engineering, to environmental remediation, catalysis, electronics, energy storage, etc [56].

Waste prevention and minimisation involves reducing waste generation and its hazardous content strictly by consuming less resources and thus reducing expenditure of recycle or disposal of wastes as well as reducing the impact on the environment. Reuse of waste implies using an item more than once, either for the same purpose or for a different purpose, refurbishing, selling or donating them to charity or community groups. Reuse is superior to recycling, as the latter is a process to transform the materials into a new product either in the old form or in a new form, which involves producing GHG from its collection to end use. However, recycling is required for the materials that cannot be reused and must be thrown away as garbage. It avoids landfilling. By separating wastes according to different materials recycle can be done efficiently.

Energy recovery from waste (waste to energy (WTE)) is also a process which transform the waste materials which cannot be recycled, into useable form, like heat, electricity, or fuel via different types of methods such as combustion, gasification, anaerobic digestion, and landfill gas (LFG) recovery.

Disposal is another type of waste processing which involves landfilling and incineration. An inoffensive landfilling is possible by appropriate design containing clay or plastic lining and proper managements landfill can be a hygienic and relatively inexpensive method of disposing waste materials [60].

The waste-to-wealth not only provide benefits to environment by significant reduction GHG emissions and avoiding use of fossil fuel, but also helps to develop new technologies, livelihoods and jobs which consequently leads to improve future lifestyle in sustainable way . [56].

2.3 Developing affordable healthcare in Low Middle-Income Countries (LMICs)

Affordability can be fundamentally defined as a function of income, expenditure, and assessments of the value of goods and services [66]. It measures the ability of someone or something to purchase a product or a service without any undesirable sacrifices. Likewise, health care affordability is the capability of a person or organisation in terms of their income, to pay for or provide for health care expenses [67]. Also, the demand or need to get healthcare service fluctuates significantly from person to person, and over time with the changing in health status [66].

Effective and affordable health care is a major problem in the low- and middle-income countries. It is evident that many people in the LMICs live without health care and are even received less health care than the rich [68]. O'Donnell *et al.* [68] reported that diarrhoea, pneumonia, and malaria causes 52% of child deaths worldwide. In South Asia, less than half of pregnant women get access to an antenatal check-up, while only one-fifth of births are supervised by trained medical person. They identified that, proper interventions may not be offered due to shortage of resources, improper distribution, and insufficient quality; while culture, illiteracy and low economic situation may negatively effect on the utilisation of the healthcare services even if it is readily available, inexpensive and its benefits are recognised [68]. Turner *et al.* [69] reported that in LMICs, approximately 8.6 million premature deaths occurred in 2016 for lack of proper facilities and timely arrival.

To rectify these facts, healthcare level and distribution in the LMICs requires to take necessary actions [68]. The difficulty in the access to the healthcare system comprises two faces: supply and demand. Lack of offering decent quality and effective health care is on supply side, whereas not using this beneficial service by individuals is on demand side. However, the two faces are linked to each other.

Poor quality care may reduce public interest, on the other hand, a high level of demand may encourage the establishment of quality care [68]. Measures should be taken for both supply and demand sides to improve the access to the healthcare system for the people of LMICs.

It is high time to develop an affordable health care services including critical care for ICU patients with the quality and availability in resource-limited settings in the LMICs. There are

very limited facilities available for critical patients in many LMICs as well. Even though many countries are moving from low to lower–middle income category, they are not having proper international healthcare aid which reduces the available resources to extend the facilities. However, developing the private healthcare systems in LMICs are partially responding the enhanced demand, their quality may vary and also be expensive for limited income people [69].

The emergence of new Innovative technologies involving affordable, sustainable and scalable models is an urgent need nowadays in the health care sectors of LMICs. The potential of new affordable technology will provide cost reduction alongside expansion of capacity and availability as well as enhancement of the care quality. Consequently, it will be possible to reduce the burden of preventable deaths in LMICs along with ensuring the basic human right of proper healthcare for the under-privileged people.

2.3.1 Bio-waste resources in LMICs.

In the LMICs, biowastes are enriched with food wastes generated mainly from the food industries and generally, they become waste without being reused, recycled or converted. Food waste is a global problem. Around 30 % of total food produced are disposed in a landfill site, which is very alarming in terms of environmental impact as well as food security. Researchers are nowadays addressing this alarming issue aiming to transform bio wastes into valuable products in a sustainable manner. Reducing food waste improves food security, boost income as well as the generates environmentally friendly secondary markets in LMICs, which also contribute to their sustainable development [70].

Though environmental pollution and the subsequent health risk due to mismanagement of wastes is a global issue, it is very critical in the LMICs, specially in the rural areas. The major obstacle for this appears to be the lack of efficient structure and regulations of the waste management sector in conjunction with low collection rates, shortage of land and high transportation costs [71]. Moreover, these areas encounter more economic and technological barriers than high income countries [72]. Communities living in rural areas also faces some additional challenges because of lack of knowledge and inappropriate tools, which creates difficulties to manage solid waste in those areas [72].

The unsustainable disposal systems, mainly noticeable in low-income countries, has severe environmental and social impacts, which hinder improvements in sustainable development [73].

The deterioration of environmental condition has adverse effect on the health of populations worldwide. Jameton *et al.* [74] reported that about 25% of health problems arise from environmental issue and at the same time, environment can be damaged by health care system. Hence, a healthy ecosystem worldwide is beneficial for human health in the long term. People worldwide need to maintain public health and provide health care in a sustainable way to get a supporting ecosystem for human health [74].

2.4 Bone tissue engineering approaches using bio wastes

Bone tissue engineering (BTE) is based on the knowledge of bone structure, bone mechanics, and tissue formation which aims to regenerate or repair damaged bone tissues via combination of biomaterials, cells, and factor therapy [75]. Bone tissue possesses self-healing properties upon the damage, fractures or minor defects, while some seriously injured tissues need clinical treatment. Bone autografts, allografts, or synthetic bone substitutes are present treatment interventions for such significant defects. However, these treatment methods have some limitations [76].

The field of bone tissue engineering has been evolved nearly four decades ago to address this issue and the successful outcomes obtained by collaborative efforts of scientists, engineers, and surgeons [76].

In this advanced biomedical research era, researchers are extensively exploring the potential use of waste-derived materials in the biomedical field considering environmental impact of wastes. Literatures reported that waste-derived bio ceramics and biopolymers possess excellent biocompatibility and biodegradability which make the bio-waste materials a prospective candidate to be used in tissue engineering [77]. The valorisation of waste materials also contributes to the minimisation of source scarcity by reducing waste disposal.

Zamri *et al.* studied the utilisation of bio-waste for tissue engineering applications and reported a wide range of bio-waste for tissue engineering applications which are mentioned below including the compound extracted from them (in bracket):

- Marine waste:
 - Cuttlefish and cod fish bone, mussel shell, fish scale and head, oyster shell (HA, β -TCP, CaPs)
 - Oyster shell cockle shell, fish head (aragonite and polysaccharide)
 - Jelly fish (collagen)
 - Prawn shell and fish processing (chitosan, gelatine and collagen)
- Plant waste:
 - Apple core and peel, corn silk, jackfruit peel, apple pomace (cellulose and pectin)
 - Gardenia jasmine flower (Genipin),
 - Curcumin and green tea leaf (polyphenols fixing agent)
- Livestock waste:
 - Eggshells and bones of chicken, bovine and pig. (HA and other CaPs)

2.4.1 Bone

The main component of the human skeletal system is bone. It is a dynamic and highly vascularised connective tissue that continues to remodel throughout the lifetime of an individual [78]. The human adult skeleton is made up by more than 206 different bones.

Bones are composed of two types of tissue [78]:

1. Cortical (compact) bone which is strong, dense and tough
2. Cancellous (trabecular) bone which is lighter and less dense

Figure 2.2 represented the anatomy of a femur bone.

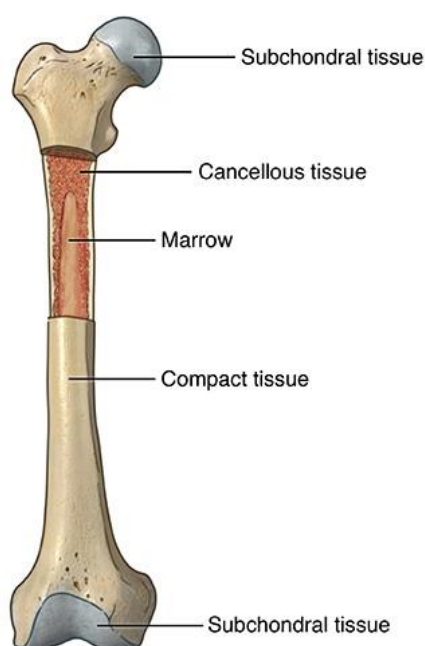


Figure 2.2: Anatomy of the bone [79].

Bone is a biocomposite which consists of around 65 wt% mineral phase, 25 wt% organic, and 10 wt% water [80]. The organic component is composed mainly of fibrous type I collagen and small amounts of proteoglycans and glycoproteins and the mineral component contains apatites, mostly hydroxyapatite [81]. Hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ is the major constituent and one of the most stable forms of biological apatite where it presents along with some traces of Na, Mg, C, Sr, F, Zn etc. The calcium to phosphorus atomic ratio (Ca/P) of stoichiometric HA is 1.6, whereas HA in biological apatite is non-stoichiometric type with Ca/P ratio of <1.67 due to the presence of traces in HA [82, 83].

2.4.2 Calcium and phosphate sources

2.4.2.1 Synthetic sources

Typically, the most conventional method to produce HA and other calcium phosphates are chemical synthesis from the inorganic components. Various synthetic chemicals has been reported in a wide range of literatures, such as CaCl_2 , $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{Ca}(\text{NO}_3)_2$, CaCO_3 or $\text{Ca}(\text{CH}_3\text{COO})_2$ were used as calcium source, whereas $(\text{NH}_2)\text{HPO}_4$, $\text{NH}_4\text{H}_2\text{PO}_4$, KHPO_4 , N_2HPO_4

and NaH_2PO_4 were utilised as phosphorus sources [84]. The key drawbacks connected with synthetically produced calcium phosphate is high cost and most importantly, it does not contain any trace of beneficial elements such as carbonate ions, Na, Mg, Sr, Zn, K, Si, Ba, F ions etc [85].

2.4.2.2 Natural sources

Calcium phosphates can also be prepared from natural by-products or wastes such as animal bones [86], eggshells [87], fish bones [88], cuttlefish shells, oyster shells [89], corals [90], wood and algae [3]. Their bioactivity and biocompatibility properties and *in vitro* applications are similar to synthetically-derived calcium phosphates. Sometimes they provide better properties than those of the latter [91]. Biological apatite, found in natural bone, is non-stoichiometric (Ca/P molar ratio <1.67) type of HA where Ca deficiency is observed and the lack of Ca is compensated by other elements such as sodium, magnesium, carbon, etc [92]. The presence of these ions directly influences various biochemical reactions linked with the bone metabolism [93]. Biological resources are generally signified as natural calcium storage and contain a wide variety of the beneficial ions in trace amount [94]. Therefore, extracting CaPs from these sources exhibit better biological properties as it possess similar inherent morphology and natural pore structure to human bone [95] and contains the valuable trace ions, which play a crucial role in the bone re-generation process and are known to speed up the bone formation process [85]. Moreover, using waste resources to prepare CaPs meet the growing need for developing clean, non-toxic and environmentally friendly techniques.

A comparison of different parameters (Ca/P, particle size, morphology, phase composition, thermal stability and trace elements) of HA produced from various natural sources are presented in Table 2.1 [48].

Table 2.1: Comparison of HA produced from various sources [48].

Parameters	Bovine bone	Fish bones	Eggshell	Natural bone
Ca/P ratio	>1.67	≥ 1.67	≥ 1.67	1.67
Particle size	Micro to nanometer range	In nanometer range	≥ 50 nm	Nanocomposite
Morphology	Needle, rod, plate, spherical, and so forth	Needle, rod, spherical, and so forth	Needle or rod	Needle or rod
Phase composition	Phase pure and tricalcium phosphate	Phase pure	Phase pure	Composite
Thermal stability	800–1000 °C	600–800 °C	<800 °C	>1200 °C
Trace elements	Na ⁺ , Mg ²⁺ , K ⁺ , Ti ²⁺ , etc	Fe ³⁺ , Cr ³⁺ , Cu ²⁺ , K ⁺ , Mg ²⁺ , and so forth	CO ₃ ²⁻ , major Element Na ⁺	Na ⁺ , Mg ²⁺ , K ⁺ , Zn ²⁺ , CO ₃ ²⁻

The production of calcium phosphates using various waste-derived calcium and synthetic chemicals as phosphorous precursors was reported in a wide range of literatures [96, 97]. However, from the environmental sustainability perspective, utilisation of a phosphate-rich waste as a phosphate precursor would contribute make the calcium phosphate synthesis process more sustainable.

Phosphorus is available abundantly in bio-waste, specially phosphorus-rich wastewater, sewage sludge, manure and waste from slaughter house and agri-food sectors [59]. These are potential sources of phosphorous. Recovering phosphorus from this waste is a growing interest nowadays as it minimises the resource scarcity with increasing nutrient recovery as well as helps moving towards sustainable world. A significant number of literatures have reported on phosphorus recovery in struvite (MgNH₄PO₄·6H₂O) form phosphorus rich wastes [98-102]. However, a very few studies have been carried out to produce calcium phosphates using both Ca and P sources from wastes [103]. For example, Kannan *et al.* [104] prepared calcium-deficient HA with Ca/P molar ratio 1.58 from bio-waste in a batch reactor. They used eggshells as a calcium source and synthetic urine (for simplicity) as a phosphorous source. In another study [103], they used same precursor materials to produce HA via an

electrochemical route. They demonstrated that calcium phosphates were formed first on an electrode (magnesium or stainless steel) and then it converted into crystalline HA after alkaline treatment at 80 °C for 2 h.

2.4.2.2.1 Eggshells as calcium source

A million tonnes of eggshells are generated as bio-wastes worldwide everyday [105]. Among them, a minor proportion is used as fertilizer or animal foodstuff whereas, a big portion is thrown as waste [106]. The eggshells thus lead to environmental pollution generating microbial growth. However, as a high-quality calcium source for the preparation of HA, the eggshells present a promising future. Consequently, recycling of eggshells helps in improving the ecosphere and reduces the cost of waste management [28].

Fresh eggshells naturally consist of a three-layered structure. The outer surface is cuticle layer which resembles a ceramic, the middle layer is spongy, and the inner layer is lamellar layers. Eggshell represents almost 11% of the weight of egg [28]. Eggshells contain around 94% calcium carbonate, 1% calcium phosphate and 4% organic matter [105]. In addition, some natural trace elements like K, Na, Mg, Si and Sr ions are present which makes the eggshell-derived HA more biocompatible to human bone which can boost the physiological function of the materials after implantation [94, 107]. Also comparing to HA synthesized from other sources eggshell-derived HA showed superior sinterability [48] and osteoconduction [108-110] and thereby benefiting the overall physiological functioning after implantation. The Ca/P ratio, particle size and morphology, phases and calcination temperature of HA powder obtained from eggshells in different literatures are presented in Table 2.2.

Table 2.2: A brief summary of the properties including Ca/P ratio, particle size and morphology, phases and calcination temperature of HA powder derived from eggshells, taken from papers reviewed by Akram et al. [85].

Paper	Ca/P ratio	Particle size (nm/lm)	Morphology	Secondary phases	Trace elements	Calcination (T °C)
1	1.65	–	–	β-TCP	–	900–1200
2	1.67	50	Rectangular	–	–	–
3	1.67	18	Spherulite	–	–	800–1200
4	–	35–50	Prolate spheroidal	–	–	700
5		78	Flower like		Mg ²⁺	
6	1.67	35	Like globules	–	–	900
7			Flower like	–	–	–
8	1.57–1.77	12–49	Needle and rod like	–	Na ⁺ , Mg ²⁺ and Sr ²⁺	–
9	2.20	–	Spheroidal	β-TCP	Na ⁺ and Mg ²⁺	1100
10	–	35–15	Flower like (microspheres)	–	–	–
11	–	0.06	Whiskers	CaHPO ₄	–	700
12	–	41	Circular	–	–	-

2.4.2.2.2 Struvite as phosphate source

Struvite (MgNH₄PO₄·6H₂O), is a P containing crystalline material which was first identified by Rawn and Banta (1939) [111] in digested sludge supernatant lines of wastewater treatment plants (WWTPs) [8, 9]. It has been reported that the blockage of pipes occurs due to struvite formation, representing a common problem in wastewater treatment plants [111]. De-Bashan and Bashan [11] proposed that P recovery through struvite formation had great potential as a slow release fertilizer and may be a promising P recovery product. Struvite contains 44% water, 39% phosphate, 10% magnesium, and 7% ammonium. It is a non-odorous, non-sludgy crystal which can release nutrients slowly. Unlike other P fertilizers, its low solubility in water helps to avoid water eutrophication problems. Therefore, struvite could be more effective for both environmental protection and nutrient recycling [12].

2.4.2.2.1 Phosphate recovery from wastewater as struvite

The conventional source of phosphorus (P) are synthetically derived chemical compounds, such as $(\text{NH}_2)\text{HPO}_4$, $\text{NH}_4\text{H}_2\text{PO}_4$, K_2HPO_4 , Na_2HPO_4 and NaH_2PO_4 . These P containing compounds are generally produced from naturally occurring phosphate rock which is a non-recyclable and non-renewable mineral resource [112]. These natural P reserves are continuously being depleted [98, 113, 114]. Bing *et al.* proposed a new phosphate rock supply model based on historical phosphorus demand and reported that phosphate rock will be depleted within the next 70 ~ 140 years for the lack of proper management [115]. Molinos-Senante *et al.* also studied economic feasibility for phosphorus recovery processes and reported that the current economically viable phosphorus reserves will be consumed within 60–70 years [116].

For sustainable development, exploring alternative resources of phosphorus is essential. Therefore, phosphorus recovery from waste materials could be another promising approach in this regard [12]. Every year, large amounts of various waste materials such as animal manures, agricultural and industrial effluents, municipal wastewater and biosolids are produced globally [111]. These wastes are enriched with higher nutrient content such as nitrogen (N) and phosphorous (P) [12]. On the other hand, the presence of P in wastewater is a key factor of water eutrophication [114]. Non-treated wastewater thus not only leads to water pollution but also contributes to loss of potentially useful resources such as N and P.

Nowadays, effective and efficient recovery of P from non-conventional P-rich sources such as wastes and residues is gaining growing attention [98, 111]. In a study by Cordell *et al.*, a high percentage of P recovery from human excreta, manure, sewage sludge incinerator ash and mixed wastewater was achieved [10]. In addition, several P recovery technologies such as biological phosphorus removal, chemical precipitation, electrolysis, adsorption and crystallisation have been developed [12, 98, 114].

Hong *et al.* [117] and Xu *et al.* [118] reported that ash from municipal sewage sludge generated by incineration also has potential for struvite recovery (with P content of 13-25% of the total mass). Kataki *et al.* reported successful struvite recovery from waste water from various industries such as tannery, textile, carmine dye, semiconductor, slaughterhouse and meat packing, food processing and yeast production [98]. They also stated that several countries such as Japan, Canada, England, USA, Australia, Germany, Netherlands and Italy

were conducting struvite recovery from food industries and municipal wastewater sludge [98].

2.4.3 Bone repair materials and procedures

Some common surgical procedures to repair bone defects are various types of bone grafting and the materials which are used for this purpose are given in Figure 2.3.

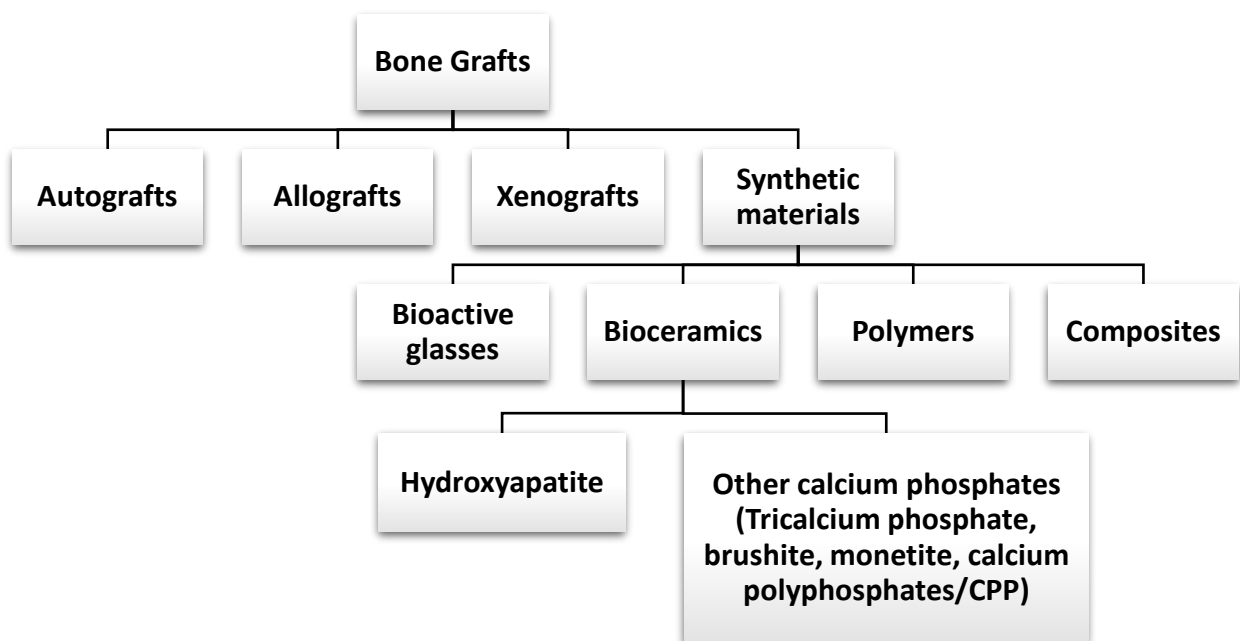


Figure 2.3: Commonly used bone repair graft materials for hard tissue regenerative applications [119].

2.4.3.1 Bone grafts

A bone graft is a biological substitute to fix problems with bones or joints almost anywhere in the body. Bone from hips, legs, or ribs can be used as a grafting material. A bone graft helps to seal an area where bone is absent or to provide structural stability. Autograft, allograft and xenograft are the categories of grafts, and their use depends on the type of injury.

Several successful surgeries have been reported for autograft, allografts and xenografts till now. However, they have some limitations which are described in Table 2.3.

Table 2.3 : Limitations of several types of bone grafts.

Bone grafts	Source	Limitations	References
Autograft	Patient's own body	• Donor site morbidity • Donor shortage • Complications in wound healing • Additional surgery is needed • Donor pain • Inadequate supply of bone to fill the gap • Additional expense	[41, 119-123]
Allograft	Another subject of the same species	• Transmission of disease • Difficulty of procurement • Premature resorption • Limited supply • Expensive • Immunogenicity	[41, 119-123]
Xenograft	Animal source	• May causes immune reaction • Inability to gain adequate height and width for large defects. • Possibilities in transferring infectious diseases	[119, 120, 122]

2.4.3.2 Bone grafts substitutes

To overcome the drawbacks of commonly used bone grafts, the development of suitable biomaterials as bone graft substitutes to promote bone regeneration has attracted a lot of interest over the last few years [123].

A bone graft substitute is a substance or combination of substances (natural or synthetic) which acts as a temporary matrix to promote tissue regeneration by providing a specific environment and architecture [124, 125]. Biomaterials are application-specific, i.e if one specific biomaterial is biocompatible for an application, it might not suitable for another application [126].

The implant material should ideally possess the following criteria [48, 122, 127-129]:

- **Biocompatibility:** This is the ability of a biomaterial to perform with an appropriate host response, in a specific application. Its properties should not be degraded (unless this is wanted) and also must not cause any adverse reaction within the host's body.
- **Bioresorbability:** The ability of a biomaterial to degrade after implantation and can subsequently be replaced by the host tissue.
- **Osteoconductivity:** The ability of a biomaterial to support bone growth.
- **Osteoinductivity:** This property helps the biomaterial to stimulate osteoprogenitor cells to differentiate into osteoblasts and then begins new bone formation.
- **High porosity:** Highly porous biomaterials allow migration and proliferation of cells, accordingly potentially facilitate tissue ingrowth. Moreover, it helps to improve mechanical interlocking between the implant material and the surrounding natural tissues.
- **Biomechanical compatibility:** The biomaterials should possess the mechanical properties required to accomplish the purpose.
- **Bioactivity:** Bone-bonding ability of a material.

2.4.3.2.1 *Bioactive inorganic materials*

The inorganic materials which are biocompatible in physiological environment and have the ability to form bond with bones are bioactive inorganic materials. They are mainly classified into two categories such as bioactive glass and bioceramics.

Bioactive glasses are amorphous in nature. The first artificial bioactive glass is silicate-based glass named 45S5 Bioglass®, which was invented by Professor Larry Hench in 1969. It was composed of 46.1 mol % SiO₂, 24.4 mol % Na₂O, 26.9 mol % CaO and 2.6 mol % P₂O₅ [130]. As like silicate glasses borate glasses also show bioactivity through the formation of bone like hydroxyapatite layers [48]. Generally, these glasses can be designed as solid or porous scaffolds [53] with highly ordered, controlled pore size and pores interconnectivity.

Bioceramics are an important subset of biomaterials. Generally, bioceramics have been used in filling and restoring bone and dental defects [131]. They are also used as coatings on metal joint prostheses, bone cement and porous scaffolds [132]. Several types of bioceramics have

been used in the fabrication of various biomaterials for the tissue engineering are classified as follows in Figure 2.4:

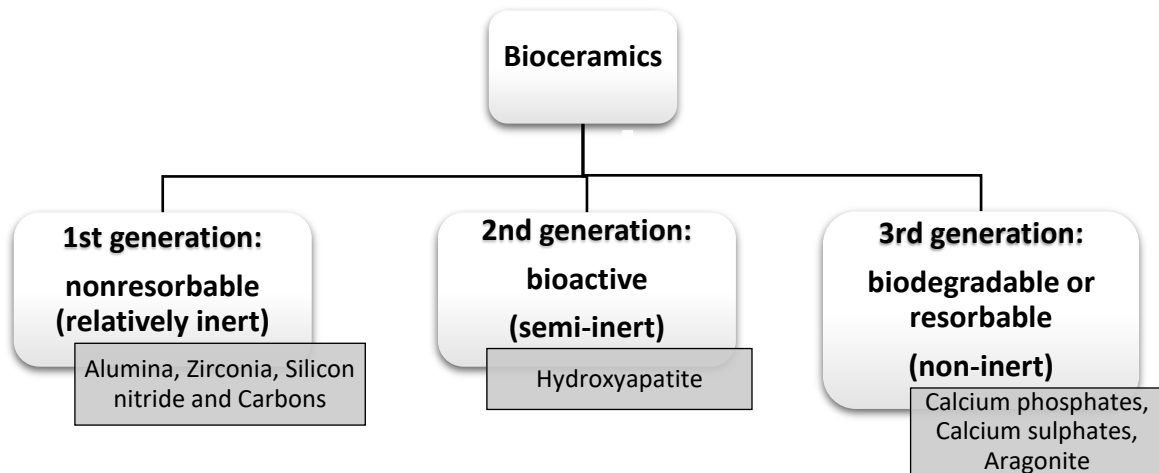


Figure 2.4: Classification of bioactive ceramic materials [56]

2.4.3.2.2 Biodegradable polymers

Biodegradable polymers are versatile class of biomaterials [133, 134]. In recent years, a large number of natural and synthetic polymers are extensively used for biomedical application [133, 135].

2.4.3.2.2.1 Synthetic Biodegradable Polymers

Synthetic polymers, which contains relatively hydrophobic materials such as the α -hydroxy acid, polyanhydrides, and others; are considered to be potential candidates in tissue engineering and regenerative medicine due to their controllable degradation rate [136, 137]. These materials showed biocompatibility, non-toxicity and biodegradability at physiological conditions for which they can be used for controlled drug release and tissue engineering [135]. It was observed that they showed significant results in case of cell viability, cell proliferation, alkaline phosphate activity and mineralization assays [75]. Moreover, they increase the strength of scaffolds and acts as a drug delivery device [127]. Synthetic biodegradable polymers are also free from immunogenicity [153].

Some commonly used biodegradable synthetic polymers for tissue engineering/regenerative medicine are poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolic acid) (PLGA), poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV), poly(dioxanone) (PDS), polycaprolactone (PCL), polyurethanes (PUs) based on LDI and poly(glycolide-co- γ -caprolactone), ethyl glycinate polyphosphazenes, poly(propylene fumarate) (PPF), poly(tartronic acid), poly(β -malonic acid), poly[1,6-bis(p-carboxyphenoxy)hexane] and tyrosine-derived polycarbonate [138, 139]. One of the major drawbacks of these polymers is their degradation times which is around half than for any of the natural polymers [127].

2.4.3.2.2.2 *Natural Polymers*

Having a diverse physical and chemical properties, natural polymers are currently used in various biomedical applications as independent biomaterials [140-142]. They have been used for treatment of bone diseases [143], arthritis [144], and so on. Both plant and animal-derived polymers, being available in nature, are biocompatible, some are biodegradable and may enhance functionalisation of the surface of ceramic scaffold to improve their bioactivity [145, 146].

Polysaccharides from various marine sources [127], collagen, alginate, gelatin, elastin, actin, keratin, albumin, chitosan, alginic acid, chitin, cellulose, silk, and hyaluronic acid are among the most widely studied biopolymers [135, 138].

The major natural polymers currently in use are proteins and polysaccharides. Their properties and applications are mentioned in Table 2.4.

Table 2.4: Commonly used natural polymers and their properties and applications [127, 135, 138, 147, 148].

Types of biopolymers	Examples	Properties and applications
Proteins	Collagen	• It is one of the body's extracellular matrix (ECM) proteins • Abundantly found in musculoskeletal tissues • Nearly 22 types of collagen exist in the human body and among them, types I-IV are the most common • It can be extracted from mammals such as rats, bovines and humans • Collagen scaffolds can be designed into desired shapes such as tubes, sponges, powders, and sheets for tissue engineering. • A wide variety of applications ranging from tissue regeneration to cosmetic surgery.
	Gelatine	• It is one of the main components of skin, bone, cartilage and connective tissues • Biodegradable and biocompatible • Due to its potential porosity, gelatine can absorb 45 times its weight in liquid • Completely absorbed into the body in 4-6 weeks • Promotes cell attachment, differentiation, and proliferation • Obtained by denaturation and partial hydrolysis of collagen • Cost-effective • Applications: ○ Pharmaceutical sector ○ Food sector

		○ To tissue engineering.
	Elastin	• Elastic and insoluble protein • Found in connective tissues • Maintain minimum platelet interaction and thus suitable for making biological coatings for synthetic vascular grafts • Applications: ○ Elastic cartilage repair ○ Bone regeneration ○ Neural tissue engineering ○ Dermal regeneration • Disadvantage is its insolubility and enhanced immune response
	Silk	• Natural silk contains two groups of proteins, fibroin and sericin • Durable, biocompatible and degradable • Surface modifications with growth factors enhances its biomedical application.
	Albumin	• Most abundant protein in human blood plasma. • Water-soluble • Maintain the blood pH and carry fatty acid in the bloodstream. • Albumins can be tailored into different forms such as nanofibers, nanospheres, and microparticles for biomedical applications • It can be used in cardiovascular and gene delivery and in production of coating materials and sutures
	Fibrin	• Helps in the blood-clotting process in the human body.

		• Possess excellent biocompatibility and degradability • The most common use of fibrin is fibrin glue for hemostasis and surgical procedures. • Fibrin can also be used as a cell carrier, drug delivery vehicle, bioactive molecule transporter and scaffolds for several tissue engineering applications in tissue engineering for skin, cartilage, bone, liver, cardiac, ocular, ligament, tendons, and nervous system
	Keratin	• Makes the outer layer of human skin, component of hair and nails. • Applications: ○ Wound healing ○ Drug delivery ○ Cardiovascular applications • Supports cell adhesion and proliferation
Polysaccharides	Chitosan	• A linear polysaccharide containing β-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine. • Properties: ○ Possesses favorable biocompatible, biodegradable and anti-bacterial properties ○ Supports in cell ingrowth ○ Osteoconductive ○ Nontoxic and non-immunogenic • Biomedical applications: ○ Drug delivery ○ Bioactive bone cement ○ Biomedical microspheres ○ Bone tissue engineering

		○ Regenerative medicine ○ Wound healing ○ Helps in controlled drug release
	Alginic Acid	• A linear polysaccharide composed of b-d-mannuronic acid and a-l-guluronic acid • Can be derived from marine brown seaweed or algae • Properties: ○ Biocompatible ○ Biodegradable ○ Low immunogenicity ○ Hydrophilic ○ Water soluble • Applications: Drug delivery • Disadvantage is its incapability to degrade in enzymatic environments and poor cell adhesion.
	Hyaluronic Acid	• Polysaccharide of alternating N-acetyl-d-glucosamine and d-glucuronic acid. • Helps for cell proliferation and migration • Biocompatible, biodegradable and Water-soluble • Applications: ○ Cartilage regeneration ○ Knee joint repair ○ They are also used as postoperative care scaffolds to heal tissues after cataract surgery ○ Contributes a significant role in regenerative medicine
	Cellulose	• The main structural component of the cell wall of green plants. • Contains several thousand d-glucose units in a polymer chain

		• Application: ○ Cartilage regeneration ○ Hard tissue regeneration ○ Development of surgical tools, dialysis membranes, and biosensors
	Chondroitin Sulfate	• Sulfated glycosaminoglycans with alternating chain of <i>N</i>-acetylgalactosamine and glucuronic acid. • Application: Wound healing

2.5 Calcium Phosphates (CaPs)

Calcium Phosphates (CaPs) were discovered in 1769 and are available in nature abundantly as phosphate ores and in living organisms. They represent the inorganic part of various calcified tissues of mammals such as bones, teeth or pathological (originated from numerous diseases), which make them significant in the field of bone tissue engineering.

2.5.1 Types and properties

They are inorganic materials consisting of three major chemical elements: calcium, phosphorus and oxygen. Also, many of them contain hydrogen in their chemical structure as an acidic orthophosphate anion (HPO_4^{2-} or H_2PO_4^-), hydroxide ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and/or incorporated water ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$). A wide range of calcium phosphates contain a varied combinations of CaO and P_2O_5 oxides (either in the presence of water or without it) and they are distinguished by the type of the phosphate anion, such as ortho- (PO_4^{3-}), meta- (PO_3^{3-}), pyro- ($\text{P}_2\text{O}_7^{4-}$) and poly- ($(\text{PO}_3)_n^{n-}$). The orthophosphates and pyrophosphates comprise multi-charged anions are identified by the number of hydrogen ions substituted by calcium ones, as for example mono- ($\text{Ca}(\text{H}_2\text{PO}_4)_2$), di- (CaHPO_4), tri- ($\text{Ca}_3(\text{PO}_4)_2$) and tetra- ($\text{Ca}_4(\text{PO}_4)_2\text{O}$) calcium phosphates.

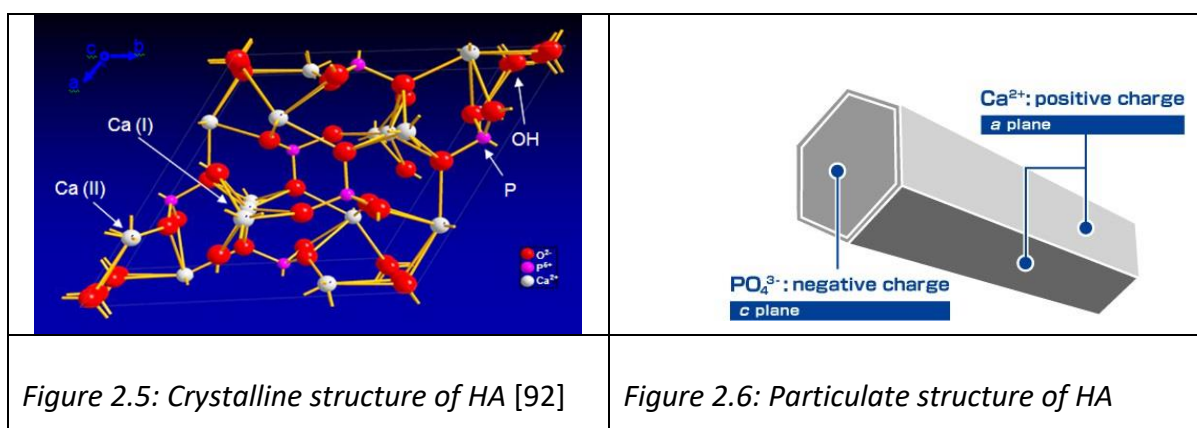
The CaPs are generally sparingly soluble in water however, readily soluble in acids but insoluble in alkaline solutions. Chemically pure CaPs are colourless transparent crystals having moderate hardness and they are white in powder form. However, the presence of impurities

and dopants, for instance ions of Fe, Mn and rare earth elements contribute to the colour of natural CaPs minerals [149].

2.5.1.1 Hydroxyapatite

Among various bioceramic materials, hydroxyapatite (HA) has attracted much attention as a fundamental substitute material [150] due to its chemical and structural similarity to the mineral components in human bone and enamel [151]. Hydroxyapatite is an essential ingredient of normal bone and teeth which provides them their hardness and toughness. Around 60-70% of bones and 90% of tooth enamel are made up with hydroxyapatite [152].

Hydroxyapatite is the most well-known crystalline phase of calcium phosphate (CaPs) [1]. HA crystals are hexagonal with cell parameters of $a=b=9.418 \text{ \AA}$ and $c=6.884 \text{ \AA}$. [92]. Generally, HA crystallites exist in different forms such as needles, rods, spheres, platelet etc. [85]. The crystalline structure and particulate structure of hydroxyapatite are presented in Figure 2.5 and 2.6.



A series of studies [153-155] showed satisfying repair function of HA artificial bones in clinical practices as it possesses following biological properties [82, 85, 94, 156-160]:

- Excellent biocompatibility
- Osteoconductive
- Promote cellular adhesion and differentiation
- Osteointegrative
- Bioactive

- Nontoxicity
- Noninflammatory behavior and
- Nonimmunogenicity

However, HA has the following weaknesses [85, 105, 161]:

- Poor mechanical properties
- Low degradation rate

2.5.1.1.1 Applications of hydroxyapatite

Hydroxyapatite is used in a wide ranges of biomedical field [92, 162] which are presented in Figure 2.7 and discussed below:

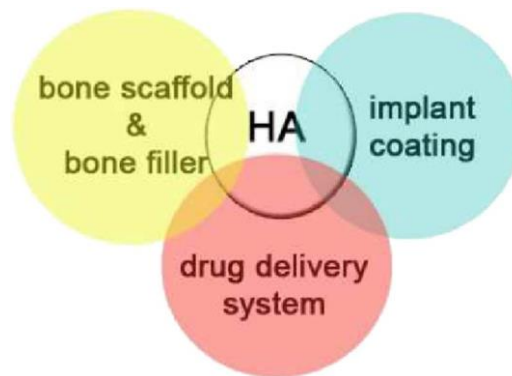


Figure 2.7: Schematic illustration of biomedical applications of hydroxyapatite [82].

- Application for hard tissue repairs [18, 162-164]
 - HA powders or granules are used for bone and tooth defect filling
 - HA particles as the component for tooth pastes and bone cements
 - Small and unloaded implants, such as in the middle ear
 - Porous scaffolds acting as temporary substrates for cell in-growth and new bone development under nonload-bearing sites
 - Coatings on metal implants for dental implants and hip joint prosthesis
 - The bioactive phase and mechanical reinforcement in a polymer-bioactive ceramic composite.

- HA scaffolds can be used for soft tissue such as skin repairs or wound healing [132, 162]
- HA materials can serve as carriers for drug/protein delivery and gene therapy [165-167]
- Applications of fluorescent hydroxyapatite nano particles in bioimaging [162, 167-169]
- HA acts as an adsorbent and purification agent in chromatography for separation of protein, nucleic acid and antibodies [162, 170]

2.5.1.1.2 Nano-Hydroxyapatite

In the era of advanced science, nanotechnology has become increasingly important for developing nanomaterials as it helps in overcoming many challenges and limitations in the use of biomaterials in tissue engineering. For example, having greater surface area than coarse crystal, nano hydroxyapatite (nHA) exhibits improved densification which may enhance their mechanical properties including fracture toughness. Moreover, nHA as an implant material shows better bioactivity and improved biocompatibility over other implants [26]. They also allow intracellular transportation and thus can be used as drug delivery systems by loading with therapeutic molecules or for imaging and diagnostics in biological systems [171, 172].

Utilizing nanotechnology, materials with unique structural and functional properties can be produced. This method helps to manipulating Ca and P at the molecular level to develop nano hydroxyapatite with a particular morphology, stoichiometry, crystallinity and crystal size distribution [105].

Matthew *et al.* reported that the ranges of bone crystals' length, width and thickness are 30–50 nm, 15–30 nm, and 2–10 nm, respectively [173]. Particle size can influence the efficiency of cell targeting, drug loading, and gene transfection. In addition, mechanical properties can be improved by using HA nano particles [174].

Advantages of nano Hydroxyapatite [18, 94, 175-178]

- Nano-crystalline HA exhibit better osteoblast functions

- n-HA possesses strong ability to bind proteins and bacteria when using in toothpaste due to their larger surface area
- Promote cell adhesion and proliferation
- Better bioactivity
- Higher resorbability
- Addition of n-HA in composites results in stiffer composites, which is able to provide higher mechanical properties leading to rapid repair of hard tissue injuries.
- Act as a carrier for drug delivery

2.5.1.2 Mg-Whitlockite

Magnesium whitlockite (Mg-WH: $\text{Ca}_{18}\text{Mg}_2(\text{HPO}_4)_2(\text{PO}_4)_{12}$) is a calcium orthophosphate in which magnesium is partly substituted for calcium [179, 180]. Mg-Whitlockite is composed of rhombohedral crystal structure with cuboid shape [180]. It is the second most abundant bone mineral in the human body which is of approximately 25–35 wt% of the inorganic part of human bone [180]. The Ca/P ratio of WH is 1.43. It is highly stable in acidic condition [180]. This compound provides higher compressive strength than hydroxyapatites and has the ability of promoting osteogenic activities simultaneously [181]. Recently, a variety of research has been carried out on Mg-WH in the field of bone regeneration. Kim *et al.* [182] studied on synthesis of Mg-WH nano-particles via wet precipitation method and demonstrated that synthetic Mg-WH nanoparticles stimulated osteogenic differentiation by prohibiting osteoclastic activity and thus helped in early-stage bone regeneration. It transformed into mechanically enhanced HA by releasing of PO_4^{3-} and Mg^{2+} continuously under physiological conditions.

A study on the development of biomimetic highly porous hydroxyapatite/whitlockite scaffolds conducted by Bauer *et al.* [183] via hydrothermal method at 200 °C and the effect of Mg on the composition and morphology of scaffolds was also studied. They used cuttlefish bone, ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) and magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) or magnesium perchlorate ($\text{Mg}(\text{ClO}_4)_2$) as starting materials to prepare hydroxyapatite and Mg-WH. They reported that the fabricated scaffolds were noncytotoxic and the scaffold containing HA and WH wt ratio of 70:30 exhibited optimum results to be

used in bone tissue engineering applications, whereas the higher mechanical properties and a higher level of the osteogenic differentiation of human mesenchymal stem cells (hMSC), was observed for the scaffolds containing 90:10 ratio of HA and Mg-WH compared to pure HA scaffolds.

2.5.1.3 α -Tricalcium phosphate (α -TCP)

α -TCP [α - $\text{Ca}_3(\text{PO}_4)_2$] is a polymorph of TCP. The IUPAC name of this compound is tricalcium di-orthophosphate alpha. This compound cannot be precipitated from aqueous solutions as it is obtained at higher temperature (above 1200 °C). It is reactive in aqueous systems and thus is stable at room temperatures in the absence of humidity. The drawback of using this material is its higher resorption rate (faster than formation of a new bone), which restricts its application in biomedical field. However, Dorozhkin reported that a biphasic silicon substituted α -TCP/HA had been commercially available as a precursor to fabricate bioresorbable porous ceramic scaffolds [149].

2.5.2 Biomedical applications of CaPs

Calcium phosphate based materials hold excellent properties of biocompatibility [184, 185], osteoconductivity [186], and osteointegration [187]. Therefore, intense investigation on these materials for biomedical application have been carried out for over half a century. This results in successful use of these types of ceramics in hard bone tissue replacement [85]. Different types of calcium phosphate ceramics differ in their chemical formula as well as the Ca/P ratio which are shown in Table 2.5.

Table 2.5: Important calcium phosphate compounds with their Ca/P ratios and applications.

Compound	Formula	Ca/P ratio	Application	Ref.
Monocalcium phosphate monohydrate (MCPM)	$\text{Ca}(\text{H}_2\text{PO}_4)_2\text{H}_2\text{O}$	0.5	Increase root fluoride uptake	[85, 188]
Monocalcium phosphate (anhydrous) (MCPA)	$\text{Ca}(\text{H}_2\text{PO}_4)_2$	0.5	Artificial bone graft	
Dicalcium phosphate anhydrous (DCPA)	CaHPO_4	1	Polishing agent for teeth, source of Ca and P in food supplements	
Dicalcium phosphate dihydrate (DCPD) or Brushite	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1	Sustained release of highly water soluble drugs	
α -Tricalcium phosphate (α -TCP)	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	1.5	Biodegradable composite for bone repair	
β -Tricalcium phosphate (β -TCP)	$\beta\text{-Ca}_3(\text{PO}_4)_2$	1.5	Orthopedic surgery	
Calcium-deficient hydroxyapatite (CDHA)	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$	1.5-1.6	Bone grafting	
Hydroxyapatite (HA)	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1.67	Repairing of hard tissues	
Fluorapatite (FAP)	$\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$	1.67	Used as source of fluorine in pharmaceutical products	
Tetracalcium phosphate (TTCP)	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	2	Applied as cements and coatings on metallic implants	
Octacalcium phosphate (OCP)	$\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$	1.33	Bone substitute materials	

2.5.3 Preparation methods of calcium phosphates

Several methods for synthesising CaPs have been evolved in the past few decades [105]. In general, they can be classified as:

- 1) Wet methods involving chemical precipitation, hydrolysis, sol-gel, hydrothermal, emulsion and sonochemical ones, ultrasonic irradiation, emulsion liquid membrane, microwave-mediated metathesis, expeditious microwave irradiation, RF thermal plasma, microemulsion and hydrolysis [94, 156].
- 2) Dry methods involving solid-state and mechanochemical (ball milling), flame-spheroidisation [87, 189, 190].

2.5.3.1 Hydrothermal synthesis

The hydrothermal synthesis method is the most widely reported as an effective and convenient process for preparing hydroxyapatite [48]. It is advantageous over other methods because the resultant hydroxyapatite exhibits high crystallinity and excellent homogeneity [106] with diverse controllable morphologies and architectures [82]. Moreover, the technology is considered as a sustainable or “green” technology as the main reaction media for this method is water, rather than toxic solvents [191].

The continuous-flow hydrothermal synthesis (CFHS) or supercritical water hydrothermal synthesis (scWHS) method utilises chemical and physical properties of superheated water to produce nanoparticles by rapid nucleation and precipitation. The properties of supercritical water helps to promote faster and more uniform mixing of reagent streams [192]. Lester *et al.* developed a novel method to produce nano hydroxyapatite with different morphologies including nanotubes via continuous flow hydrothermal synthesis. A series of different samples were prepared through altering the experimental temperatures, concentration and the pH of the precursors. The temperature was varied between 200 and 400 °C and pressure of the system was maintained at 240 bar. In this study, they established that the more favourable temperature to manufacture hydroxyapatite nanoparticles was 200 °C [193].

Elizondo-Villarreal *et al.* used eggshells as the source of CaCO_3 for the synthesis of hydroxyapatite by hydrothermal method [106]. CaO , obtained from direct calcining of the

eggshells, was reacted with commercial calcium dibasic phosphate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$). The final product obtained was a mixture of hydroxyapatite and CaHPO_4 in a 3:1 ratio. The morphology of HA obtained was in whiskers form which is suitable for dental prosthesis applications [106].

Shih-Ching Wu *et al.* also synthesised nano HA from various wastes such as, eggshell and three kinds of fruit waste extracts: grape, sweet potato, and pomelo peels. From this study, high-purity hydroxyapatite (HA) nanoparticles containing several important trace elements, such as Na, Mg and Sr was obtained from all of the sources using the hydrothermal method. Reaction temperature was maintained at 150 °C for 24 and 72 hrs in independent experiments. The results revealed that shape, size and morphology of HA crystal were affected by hydrothermal reaction times and amount of biomolecule. In case of 24 hr experiment, tiny needle-like or rod-like nano-structures were found, while a transformation from needle-like to rod-like nanostructures was observed for 72 hrs reaction. The structure of HA produced from pomelo peelings showed more resemblance with the structures of natural human bone [3].

The wet chemical methodologies are complicated and time consuming. Moreover, they need adjustment and control of pH to produce hydroxyapatite. Due to these shortcomings of the wet synthesis methods, the dry process is considered to be more suitable for the high crystalline HA synthesis. In addition, dry methods provide high reproducibility and low processing cost [87, 189].

2.5.3.2 Ball milling

In 2011, Samina Ahmed *et al.* successfully prepared pure and doped hydroxyapatite from eggshells by using solid-state method for the first time [190]. The mixture of requisite amount of eggshell powder and $(\text{NH}_4)_2\text{HPO}_4$ were ball milled for about 8 hrs. Ca/P ratio was maintained at 1.66. ZnO and NaF were chosen as doping materials due to the diverse role in biological functions of zinc and fluoride ions. The ratio of doping precursor and eggshell powder was 1:10. Each of the mixture obtained from ball milling was calcined at 900 °C for 30 minutes at 3 °C / min rate [190].

Eggshell derived nanocrystalline hydroxyapatite was successfully prepared by Ramesh *et al.* via direct solid-state sintering method. The mixture of calcined eggshell powder and dicalcium

hydrogen phosphate di-hydrate was heat treated at 800 °C and flower-like HA powder was produced. It was sintered at various temperatures ranging from 1050 °C to 1350 °C for 2 hrs. Study on the thermal stability and the effect of sintering temperature on the microstructure as well as properties of the resulting HA showed that the HA phase decomposed to tri calcium phosphate and tetra- calcium phosphate after sintering beyond 1250 °C [194].

Shih-Ching Wu *et al.* [195] demonstrated synthesis of hydroxyapatite using dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, DCPD) and eggshell powders through ball milling and heat treatment. The result showed that after 1 hr milling of the samples followed by heat treatment at 1000 °C for 1 hr formation of HA phase were initiated. Biphasic calcium phosphate (HA + β -TCP crystals) was obtained by ball milling for 5 hrs, whereas pure HA was formed after 10 hr milling at same sintering conditions. FTIR analysis revealed that the carbonate peaks of the as-prepared HA closely matched those of the biological apatite. The elemental composition of the synthesized HA confirmed the presence of Ca, P, Mg, and Sr [195]. They also studied the effects of heat treatment on the hydroxyapatite synthesis from eggshell powders and DCPD via ball milling [94]. The ratio of the starting materials was maintained at 4:3 (mole ratio). Eggshell powder and DCPD were homogeneously mixed with deionized water prior to wet-milling in a planetary ball-mill for 1 hr at a speed of 170 rpm. After drying the slurry at 150 °C for 24 hrs, the sample was heat-treated. Heat treatment was performed by varying the temperature (900, 1000, 1100 or 1200 °C) and durations (1, 3, 5 or 10 hrs) at a fixed rate of 10 °C /min in several independent experiments. It was revealed that milling the starting materials for 1 hr followed by sintering at 1200 °C for 1 hr produced HA with high phase-purity. Also, biphasic calcium phosphate (HA and β -TCP) could be prepared by varying the heating temperature and milling time [94].

Another investigation on calcium phosphate bioceramics synthesised from eggshell powders was done by Wen-Fu Ho *et al.* via solid state reaction followed by heat treatment. Single hydroxyapatite (HA) phase was obtained from calcined eggshell powders (CaO) and DCPD when heat treated at 1150 °C for 3 hrs. No diffraction peaks of starting materials and no β -TCP were found by XRD analysis. On the other hand, almost pure β -TCP with a trace amount of HA was produced from raw eggshell powders (CaCO_3) and $\text{Ca}_2\text{P}_2\text{O}_7$ after heat treatment at 1100 °C for 3 hrs [87].

Synthesis and characterisation of eggshell-derived hydroxyapatite via mechanochemical method was studied by Hamidi *et al.* In this study, the chemical reaction between calcined eggshells and dicalcium hydrogen phosphate dihydrate (DCPD) or phosphoric acid (H_3PO_4) was involved. Crystallite size and particle sizes of HA was obtained in the range of 8–47 nm and 250–550 nm, respectively. It was observed that the phase purity of HA increased with the increase in milling rotational speed. Higher degree of crystallinity of HA and the appearance of β -tricalcium phosphate (β -TCP) as secondary phase resulted with the higher sintering temperature of HA samples [196].

2.5.3.3 Flame spheroidisation process

The flame spheroidisation process is a relatively fast and inexpensive process. The outcome of sphere size and shape can be affected by several parameters of the technique; such as i) particle separation according to a specific size range before entering the flame is a key criterion to obtain dispersed uniform spheres; ii) residence time in the flame is also an important factor as larger particles will require a longer residence time for the glass to spheroidise, iii) the flame temperature can also influence outcomes of the microspheres produced and is generally controlled by the type of fuel and ratios used [197]. A variety of gases have been utilized in several studies to generate a flame which includes propane/oxygen, acetylene/ oxygen, petrol/oxygen [198] and natural gas/air flames [197, 199, 200].

2.5.4 Microspheres

In recent years, microspheres are gaining much attention to be utilised in the treatment of damaged tissues, such as bone, cartilage, and muscle. Ceramics, glasses and polymers are employed to prepare microsphere, of which ceramic and glass-based microspheres are widely considered for bone tissue regeneration, dental and orthopaedic applications [30]. Microspheres can be manufactured with external or internal porosity, a combination of both and with or without interconnectivity [31]. Porous microspheres are beneficial due to their greater surface area and lower mass density [31] which subsequently exhibits enhanced cell

attachment, cell proliferation, drug absorption and drug release kinetics compared to bulk microspheres [30].

The advantages of microspheres over particles are as follows [30]:

- They can be produced in uniform size and shape which helps them to be delivered to the specific target site.
- Porous or hollow microsphere can be manufactured which allows encapsulation of biomedically relevant components.
- A larger surface area of porous microspheres permits for adequate therapeutic coatings and promotes degradation rate and ion release.

The applications of microspheres in biomedical fields includes drug carrier, direct filling materials for tissue augmentation. Generally granular form of bioceramics (calcium phosphates and bioactive glasses) are used in the dental filling and bone regeneration. The microspheres containing macropores showed better performance as cell carriers which facilitate vascularization, nutrient supply and delivered sufficient cells. Moreover, microspheres are an excellent injectable system for minimally invasive surgery [201]. Microspheres can be prepared by various processing techniques such as sol-gel, flame spheroidisation, tube furnace method etc [30]. Wu *et al.* [201] studied on developing porous calcium-phosphate microspheres from calcium-deficient hydroxyapatite (d-HA) powders via a water-in-oil emulsion technique followed by sintering at 700, 1100, 1200, or 1400 °C for 6 h. Camphene was used as the porogen. They reported that the developed microspheres contained interconnected porous structure with pore sizes ranging between several microns to 250 µm of which around 35-50 % of the pores were greater than 100 µm. Pure HA, β-TCP along with HA, combination of α-TCP, β-TCP and small amount of HA and α-TCP along with HA were obtained by sintering the microspheres at 700°C, 1100, 1200 and 1400 °C respectively.

A simple wet-chemical method was used to synthesise porous CaPs microspheres at room temperature using fructose-1,6-bisphosphate (FBP) as the phosphorus source by Liu *et al.* [33]. They demonstrated that the prepared CaPs porous microspheres possessed higher biocompatibility and promoted the osteogenic differentiation of MC3T3-E1 (clonal murine cell line of immature osteoblasts derived from mice) and could be used as an anti-

osteosarcoma drug carrier and osteoinductive material for bone regeneration in the treatment of osteosarcoma.

2.6 Chitosan (Ch)

Chitosan, a versatile biopolymer, is a derivative of chitin which is found in the hard exoskeletons of crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as aspergillus and mucoshrimp and crab [202]. It is derived by deacetylation of chitin [(1→4)-2-acetamide-2-desoxy-β-Dglucan] (see Figure 2.8) which is one of the most abundant and inexpensive polysaccharides found in nature. Chitosan possesses favorable biocompatible, biodegradable, osteoconduction and anti-bacterial properties [127, 147].

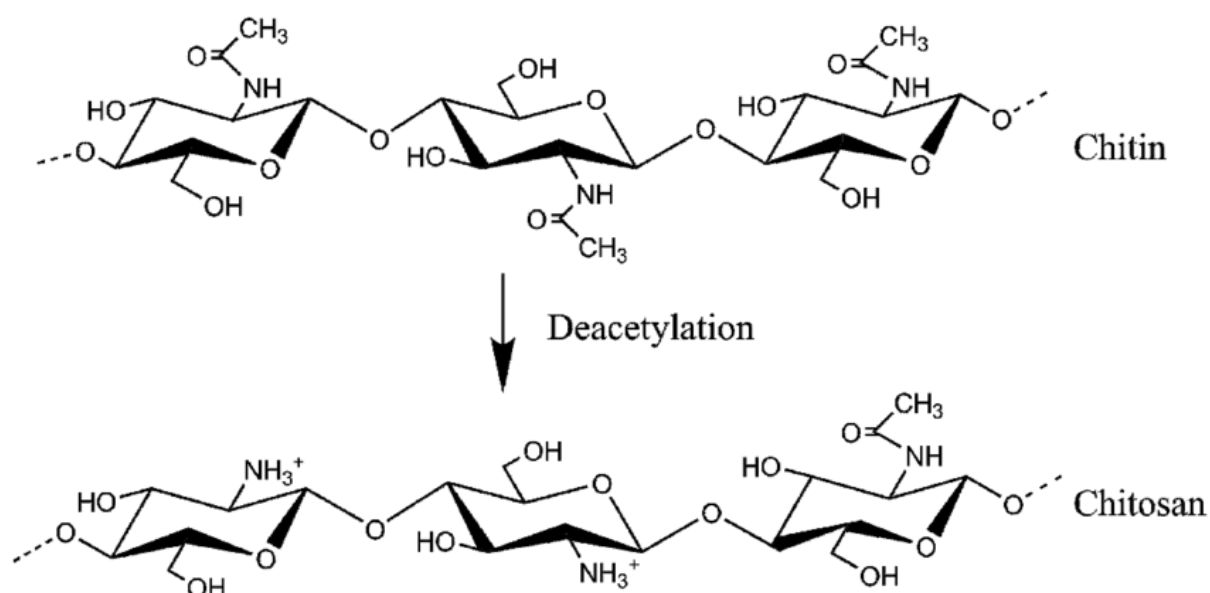


Figure 2.8: Molecular structure of chitosan [203]

Chitosan is a biodegradable cationic amino polysaccharide [127]. It is soluble in diluted acidic solutions (pH < 6.0) where glucosamine amino free groups become protonated [204]. Molecular weight of chitosan is varied from 300 to 1000 kDa with a deacetylation degree (DD) of 60 to 95% depending on the source and preparation method [127]. One of the major features of chitosan is that it is beneficial to improve cell adhesion compared to other synthetic polymers. Moreover, the positive charges on chitosan surface help to bind negatively charged growth factors and cell membranes to a greater extent [153, 205].

Chitosan presents ease of processability, controllable mechanical properties, availability of reactive functional groups and negligible foreign body rejections [41, 138]. Chitosan can be tailored into different forms such as sponges [206], hydrogels [207], fibers [208], films [209], membranes [210] and scaffolds [211] by various processing methodologies. Freezing and lyophilizing of chitosan solution produce porous chitosan structures [127] which have a highly significant role in the bone implantation process.

Several methods have been used to prepare chitosan nanoparticles, such as ionotropic gelation, emulsion cross-linking, emulsion-solvent extraction, emulsification solvent diffusion, emulsion-droplet coalescence, complex coacervation, reverse microemulsion technique in addition to self-assembly [148].

Major shortcomings of chitosan are its low mechanical properties and insolubility in water, which can result in the brittle nature of the material [138].

By blending chitosan with other natural and synthetic polymers desired properties can be achieved [138, 212]. Combination of chitosan with different materials such as hydroxyapatite, alginate, hyaluronic acid, calcium phosphate, poly (methyl methacrylate), poly-L-lactic acid and growth factors can develop new composite materials for orthopaedics [127].

2.7 Calcium phosphates (CaPs) - chitosan (Ch) composites

Next generation biomaterials are looking at combining bioactive and bioresorbable materials, which mimic the natural function of bone and activate *in vivo* mechanisms of tissue regeneration [92]. Nowadays a significant amount of research has been focused on the development of ceramics and polymer based porous scaffolds for bone tissue engineering [26, 92, 127, 213]. Inorganic ceramic phase contributes to the mechanical strength as well as toughness of the composites. Furthermore, being biocompatible and osteoconductive [187], calcium phosphates, specially crystalline hydroxyapatite enhance the attachment, growth, and proliferation of human osteoblast cells to the bioactive polymer matrix. Hence the incorporation of hydroxyapatite into a polymeric matrix is of great interest [82].

Chitosan is an excellent biopolymer frequently used to prepare composite with calcium phosphate. As chitosan potentially presents biocompatible and biodegradation properties

composite containing chitosan effectively supports orthopaedics and dental applications [135].

The ideal properties of a composite can be summarised as follows [80, 213]:

- Provides appropriate shape and volume
- Should be compatible with the surrounding biological fluids and tissues in order to minimise the immunological response
- Should be non-toxic
- Should possess a suitable degradation rate
- Should provide temporary but optimum mechanical support at the implantation site
- It should also be able to carry and deliver cells and biosignals
- Should exhibit favourable surface properties for cell attachment and differentiation

2.7.1 Types of composites and fabrication methods

In literature, a numerous types of chitosan-calcium phosphate composites have been reported, such as pastes, coatings, particles, films, scaffolds and hydrogels for orthopaedic and tissue engineering applications [42]. The fabrication methods of chitosan-hydroxyapatite composites for orthopaedic and tissue engineering applications varied according to the type of structure [42]. For the preparation of composites, mixing of composite solution is carried out generally using magnetic stirrer before performing freeze drying method. Resonance acoustic mixing is a newly developed mechanochemical mixing method which can be used to mix the composite solution in rapidly. Different form of composites and their preparation methods are presented in Table 2.6.

Table 2.6: Different form of composites and their preparation methods [42, 204, 214, 215]

Form of composites	Preparation Methods
Paste	Manually kneading (with a spatula) of HA containing powder mixture into a chitosan solution at room temperature.
Coatings	Spraying, electrophoretic deposition, electrochemical Deposition, sol-gel process
Particles	Spray-drying, Precipitation, in situ hybridisation
Hydrogels, films/membranes	Precipitation followed by casting
Scaffolds	Freeze-drying

2.7.1.1 Scaffolds

Tissue engineering aims to develop replicates of native tissue which is able to promote regeneration of damaged tissue. Fabricating three-dimensional (3D) scaffolds provide an excellent choice to serve this purpose in bone tissue engineering field and thus has been extensively investigated nowadays. The porous structures of scaffolds allow adequate transportation of blood and nutrient as well as provide suitable environment for tissue regrowth which allow them to consider as potential implant materials to be used in bone repair [46, 214, 216].

Ressler *et al.* [217] synthesised HA from hen eggshells, seashells and cuttlefish bone and then prepared HA-ch composite scaffold by freeze-gelation technique. The synthesised powders were nano-structured calcium deficient HA and amorphous calcium phosphate (ACP) and contained Sr^{2+} , Mg^{2+} and Na^{+} ions which are important for bone growth. The fabricated scaffolds were composed of highly porous structures with interconnected pores and homogeneously distributed HA particles and scaffolds showed good viability and proliferation of seeded cells with no toxicity. Zhou *et al.* [218] synthesised HA and WH hollow microspheres via microwave-assisted hydrothermal method. Then, HA-Ch and WH-Ch composite scaffolds were fabricated by freeze drying to compare their effects on bone regeneration in calvarial defects in a rat model. They reported that the porous WH-Ch scaffolds promoted bone regeneration in calvarial defects significantly and WH-Ch scaffolds could be more promising for applications in tissue engineering compared to porous HA-Ch scaffolds.

2.7.1.2 Films

A variety of composite films have been fabricated in tissue engineering, however, application of this form of composite is comparatively limited in bone tissue engineering field. Wang *et al.* [219] investigated the mechanism of chitosan-collagen composite film mediation of MC3T3-E1 cell differentiation. The outcome of their study showed that the chitosan-collagen composite film accelerated osteoblast proliferation, differentiation and matrix mineralisation in MC3T3-E1 cells, which revealed the potential use of this film in bone tissue engineering field.

Kim *et al.* [47] prepared a highly flexible HA-Ch composite film using homogeneously dispersed solutions of microscale HA powder and chitosan at various concentrations. They reported that up to 70 % of HA could be incorporated to have chitosan-based films without microcracks, phase separation and brittleness. This film possessed well distributed HA in the structure and good physio-mechanical properties. They anticipated that these films would be potential carrier to deliver HA in the localised osteoporosis area via a patch on bone.

2.7.1.3 Resonance acoustic mixing (RAM)

Resonance acoustic mixing (RAM) is a new technology designed for intensive mixing of particles. It has ability to minimise the damage of impact-sensitive material's particles such as explosives. In resonance acoustic mixing (RAM), a sample vessel is positioned on a plate which is linked to a bed of springs. This set-up is then forced to oscillate at a fixed mechanical resonance frequency of the device. This motion is transmitted to the materials which introduce intense mixing [220]. A very few research have been conducted using RAM so far. In 2021, Troksa *et al.* [221] developed mixed ceramic additive manufacturing ink with spherical zirconia grinding beads (4 mm diameter) in a LabRAM II acoustic mixer for 3 h at 70 g-force to break down agglomeration and disperse the particles. To ensure homogeneous dispersion, the mixing time was optimised by SEM images of the inks at different time points [222].

2.7.1.4 Freeze drying

Freeze-drying (or lyophilisation) is a conventional technique for the fabrication of 3D scaffold. It assists to design a complex scaffold geometry with consistent pore morphology [223]. Solutions are converted into solids in four steps: pre-treatment, freezing, primary drying, and secondary drying. The first step involves stabilisation of precursor solution to be ready for the freezing step, the solution is then cooled down to a temperature below the triple point of the solvent in the second step to sublime the solvent crystal and to form an orderly network within the interstitial spaces of polymer [224]. The final drying phase contains two separate steps of sublimation and evaporation in which the solvent in frozen and unfrozen components are eliminated, respectively. To attain a controlled interconnected porous network while avoiding architectural damages of scaffolds, temperature and pressure cycles are adjusted

accurately. The limitation of this technique is that it consumes energy and time significantly [225].

2.7.2 Crosslinking of composites

Chitosan based composites may not exhibit significant mechanical strength [46, 226]. In addition, loss of mechanical strength and integrity of plain chitosan as an implant also occurred due to water absorption and swelling [226]. To overcome these shortcomings, crosslinking can be carried out. There are two types of crosslinking methods used for crosslinking, such as chemical crosslinking and photo-crosslinking. The presence of the -OH and -NH₂ groups in chitosan structure allow it to form hydrogen bonds with other polymers [46, 226].

2.7.2.1 Different types of crosslinker

Crosslinkers are of two-types, such as synthetic crosslinker and naturally derived crosslinker. For example, glutaraldehyde and formaldehyde are synthetic crosslinker and have been widely used to increase the stability of chitosan composite scaffolds. However, these crosslinkers may cause toxicity after degradation or while liberating non-reacted groups. This limitation of synthetic crosslinkers has led to focus on using enzymatically or naturally derived cross-linking agents [227]. Genipin is a natural originated crosslinkers which are nowadays extensively used in the biomedical research [214].

2.7.2.1.1 Genipin

Genipin is a naturally derived cross-linker that is extracted from *Gardenia Jasminoides* Ellis fruit and flowers via hydrolysatation. It is a stable compound, has a small molecular weight and exhibit excellent cross-linking properties. Most importantly, genipin provides a noncytotoxic environment for various amine group containing polymers, such as protein, collagen, and chitosan [77]. Moreover, genipin cross-linked composites has ability to reduce any defects in the biomaterials which are commonly observed in scaffolds. For example, Lu *et al.* reported that the genipin crosslinked hydroxyapatite/hydroxypropyl chitosan composite scaffolds had controlled porous structure due to the reduction of hydrophilic nature, which prevented the scaffolds from degradation [77].

Pitrolino *et al.* [214] developed genipin cross-linked chitosan-nHA bioresorbable scaffolds using combined method of freeze drying and porogen-leaching, where polycaprolactone (PCL) microspheres were used as a porogen. They reported that the fabricated scaffolds showed controlled porosity and pore size distribution with excellent mechanical properties and supported early-stage osteogenesis and chondrogenesis which made them potential candidate for damaged cartilage and bone regeneration.

Chapter 3

Materials and Methodologies

3.1 Materials

In this study, the precursors used were eggshells, diammonium hydrogen phosphate $[(\text{NH}_4)_2\text{HPO}_4]$, calcium nitrate tetrahydrate $(\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O})$ and struvite $(\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O})$. Ammonium hydroxide (NH_4OH) and sodium hydroxide (NaOH) were used as pH buffer. Nitric acid (HNO_3) and acetic acid (CH_3COOH) were used to dissolve waste-derived precursors (eggshells and struvite). Chitosan (molecular weight of 471 kDa and degree of deacetylation of $84\% \pm 2\%$) from Weifeng Kehai Ltd was used to fabricate chitosan-based composite. Genipin was purchased from Guangxi Shanyun Biochemical Science and Technology Co. Ltd, China (98% purity) and used as a crosslinker of the composite scaffolds. Phosphate-buffered saline (PBS) tablets from Thermo Fisher Scientific (Loughborough, UK) were used for the swelling test of the scaffolds. Eggshells were collected from household wastes, struvite was purchased from Alfa Aesar (UK) and the other chemicals used were obtained from Sigma Aldrich, UK. Nano-hydroxyapatite rods (nHA, 30–40 nm diameter and 100–200 nm length) were from a 14.5% (w/v) water suspension obtained from Promethean Particles Ltd [228] and used to in scaffold fabrication.

3.2 Methodologies

3.2.1 Experimental Procedure

3.2.1.1 Pre-treatment of eggshells

The eggshells collected were pre-treated by first removing the membranes manually and then boiling them in water at 100 °C for 30 min. After drying them overnight, eggshells were ground using a planetary ball mill (RETSCH PM 100, Germany) at 500 rpm for 10 min.

The steps involved in the pre-treatment of raw eggshells are presented in Figure 3.1.

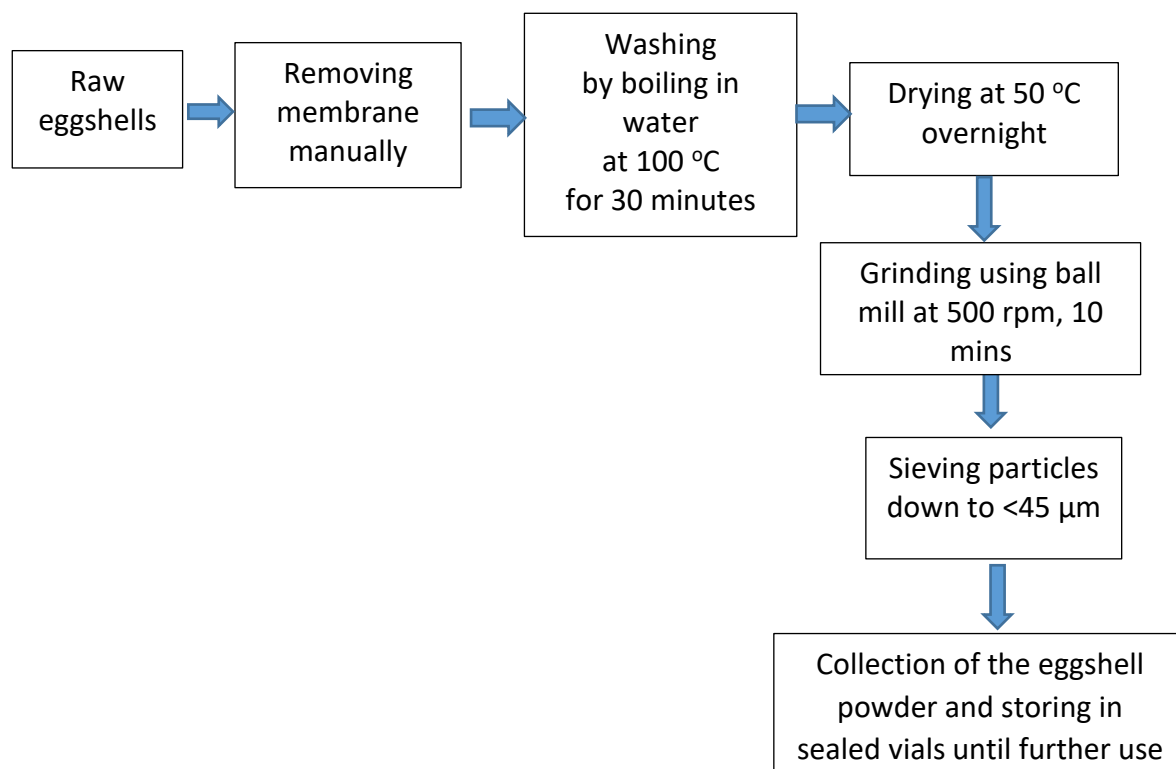


Figure 3.1: The steps involved in pre-treatment of the eggshell materials collated for hydroxyapatite (HA) or Calcium phosphates (CaPs) synthesis.

After grinding, the eggshell powder was sieved to less than 45 μm to get a uniform fine powder. Using the eggshell powder, the range below 45 μm, as a precursor, calcium phosphates (CaPs) production was trialed via three methods:

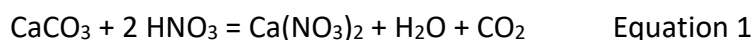
- 1) Hydrothermal process
- 2) Ball milling followed by sintering
- 3) Ball milling followed by flame spheroidisation

3.2.1.2 Continuous flow hydrothermal synthesis

3.2.1.2.1 Eggshell solution preparation

To prepare 0.05 M 1L eggshell solution, 5.15 g of ground eggshell powder (<45 μm) was dissolved in Conc. HNO₃ with a ratio of 1:2.5 (w/v). The undissolved substances of eggshells were removed by filtration and the resultant solution was made up to 1L of de-ionised

water. This solution with approximately 0.05 M concentration of Ca^{2+} ions was used as a stock solution for further experiments.



Eggshell solutions of pH 5, 8 and 10 were prepared by using two different base solutions namely NH_4OH and NaOH .

Another batch of eggshell solutions of the same concentration and volume was prepared maintaining the same protocol using acetic acid (CH_3COOH) instead of HNO_3 to dissolve the eggshell powder with a ratio of 1:3.63 (w/v) and NH_4OH used to adjust the pH of the solution.



Eggshell solutions dissolved in nitric acid were denoted as 'E(N)' and those dissolved using acetic acid presented as 'E(Ac)'. The pH of the solutions adjusted using NH_4OH and NaOH were labelled with 'Am' and 'S' in the respective codes. The numbers in brackets within the sample codes represent the pH of the solutions used.

3.2.1.2.2 Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) solution

A solution of 0.05 M calcium nitrate tetrahydrate was prepared by dissolving it in deionised water. The pH of the solution was maintained at 10-11 using NH_4OH and NaOH .

Samples prepared using calcium nitrate tetrahydrate have been labelled with 'Ca' in their respective codes.

3.2.1.2.3 Diammonium hydrogen phosphate $[(\text{NH}_4)_2\text{HPO}_4]$ solution

A 0.015 M solution of $(\text{NH}_4)_2\text{HPO}_4$ was prepared in deionised water. The pH of as prepared solution was 8 and solutions of pH 10 were prepared using the same base solutions (i.e. NaOH and NH_4OH).

The samples using $(\text{NH}_4)_2\text{HPO}_4$ as a phosphate source have been labelled with 'P' in their respective codes and the numbers in brackets within the sample codes represent the respective pH of the solutions used.

3.2.1.2.4 Struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) solution

To prepare 0.015 M struvite solution, struvite was dissolved in deionised water followed by dropwise addition of HNO_3 with continuous stirring, until a clear solution was obtained. The pH of these solutions was kept at 6, maintained using NH_4OH and NaOH individually. In addition, another batch of struvite solutions was prepared by dissolving it in acetic acid (struvite : acetic acid = 1:3) instead of nitric acid following the same procedure mentioned above for nitric acid. Only NH_4OH was used to adjust the pH.

Samples prepared using struvite have been labelled with 'St' in their respective codes. Labelling for acids and bases were kept same as mentioned above.

3.2.1.2.5 Hydrothermal synthesis process

Samples were prepared via a continuous hydrothermal synthesis route using a symmetrical counter-current nozzle reactor (see Figure 3.2 and Figure 3.3) [228]. Eggshell solutions and calcium nitrate solutions were used as calcium (Ca) sources. Whilst the diammonium hydrogen phosphate solutions and struvite solutions were used as the phosphate (P) sources. Samples were produced at varying pHs to investigate the influence of pH of solutions on the morphology of nHA/CaPs produced.

Hydrothermal synthesis process was carried out in 3 different sets of experiments using the following combination of Ca and P sources:

- a) Eggshells – diammonium hydrogen phosphate
- b) Calcium nitrate tetrahydrate – struvite
- c) Eggshells - struvite

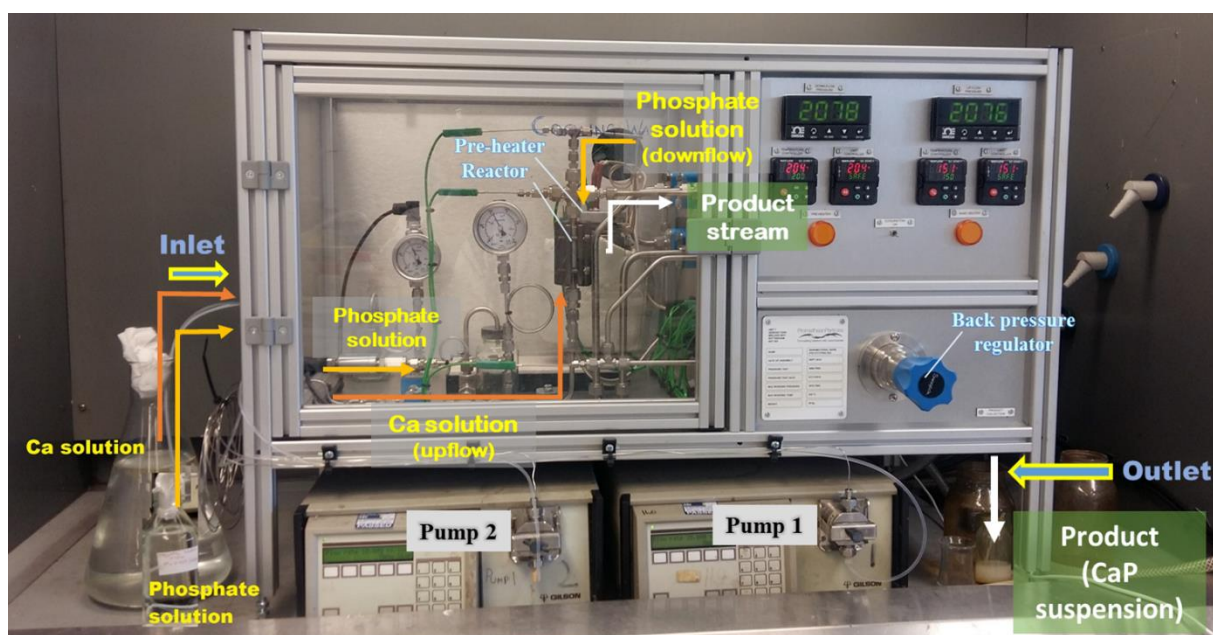


Figure 3.2: Continuous hydrothermal unit for hydroxyapatite/CaPs synthesis.

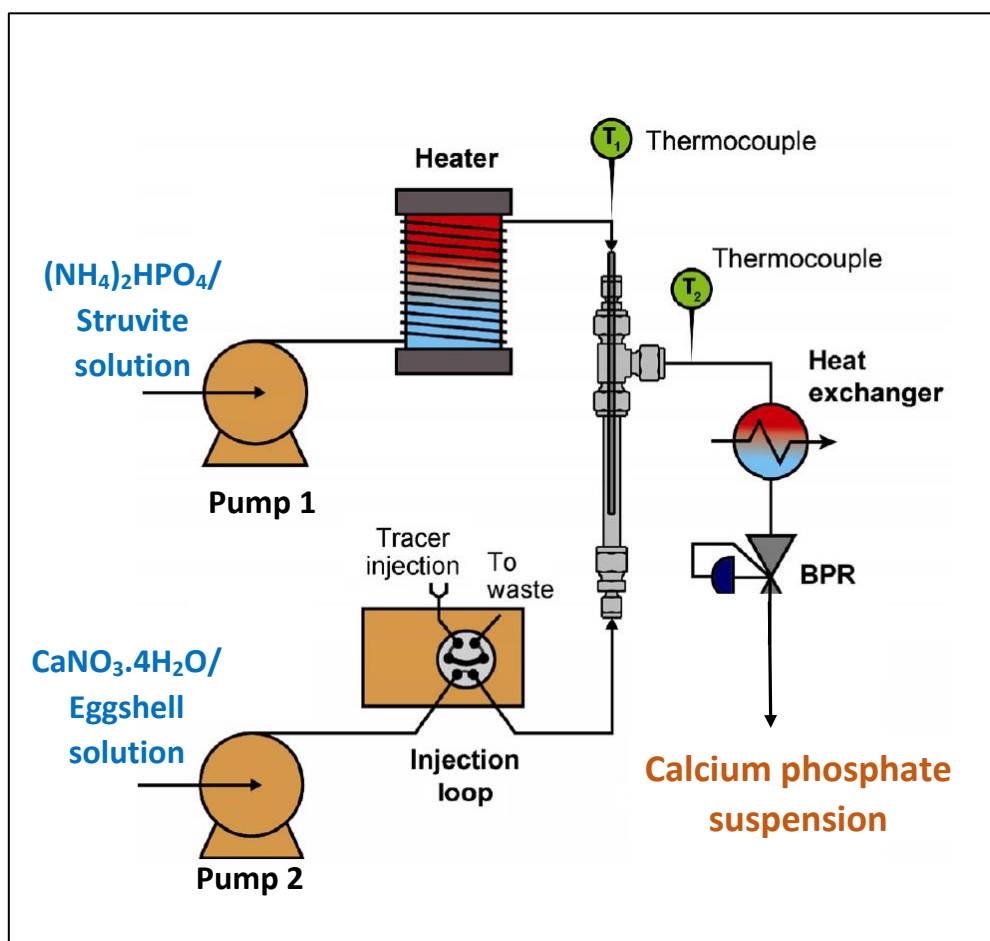


Figure 3.3: A schematic diagram displaying the reactor setup for the synthesis of hydroxyapatite/CaPs [229].

3.2.1.2.5.1 Samples prepared using eggshells as Ca and $(\text{NH}_4)_2\text{HPO}_4$ as P sources

Eggshell solution was pumped (10 ml/min) into the reactor as up-flow. Simultaneously, 0.015 M $(\text{NH}_4)_2\text{HPO}_4$ solution, was also used and introduced as the down-flow solution in the system, which was pumped (at 20 ml/min) through a preheater to the reactor chamber. The temperature was also maintained at 200 °C and 350 °C (as highlighted in sample codes) which were measured using a thermocouple and the pressure was kept at 240 bar with the use of a back-pressure regulator.

Initially, 22 different samples were prepared under different experimental conditions to investigate the effect of precursor solution pH, changes from use of different base solutions (NH_4OH and NaOH which were used to maintain pH of the precursor solutions) and temperature on the morphology of the samples produced. To simplify the results, the samples produced were separated into four groups as shown in Table 3.1, 3.2, 3.3 and 3.4.

Table 3.1: Samples were synthesised via hydrothermal synthesis process at 200 °C at different pH (5, 8 and 10) of eggshell solution adjusted using NH_4OH and NaOH and at a constant pH 8 of the diammonium hydrogen phosphate solution (as prepared). E(5/8/10)=eggshell with corresponding number representing pH of this solution, P(8)=diammonium hydrogen phosphate at pH 8, Am = NH_4OH and S = NaOH .

Samples Codes	Eggshell solution (E = Ca source)		Diammonium hydrogen phosphate solution (P = P source)
	pH	Reagent for pH control	pH
E(5)Am-P(8)	5	NH ₄ OH	8 (as prepared)
E(8)Am-P(8)	8		
E(10)Am-P(8)	10		
E(5)S-P(8)	5	NaOH	
E(8)S-P(8)	8		
E(10)S-P(8)	10		

Table 3.2: Samples were synthesised via hydrothermal synthesis process at 200 °C at different pH (5, 8 and 10) of eggshell solution adjusted using NH₄OH and NaOH and at a constant pH 10 of the diammonium hydrogen phosphate solution adjusted by using NH₄OH. E(5/8/10)=eggshell with corresponding number representing pH of this solution, P(10)=diammonium hydrogen phosphate at pH 10, Am = NH₄OH and S = NaOH.

Sample Codes	Eggshell solution (E = Ca source)		Diammonium hydrogen phosphate solution (P = P source)
	pH	Reagent for pH control	pH
E(5)Am-P(10)Am	5	NH ₄ OH	10 (adjusted by using NH ₄ OH)
E(8)Am- P(10)Am	8		
E(10)Am- P(10)Am	10		
E(5)S- P(10)Am	5	NaOH	
E(8)S- P(10)Am	8		
E(10)S- P(10)Am	10		

Table 3.3: Samples were synthesised via hydrothermal synthesis process at 200 °C at different pH (5, 8 and 10) of eggshell solution adjusted using NH₄OH and NaOH and at a constant pH 10 of the diammonium hydrogen phosphate solution adjusted by using NaOH. E(5/8/10)=eggshell with corresponding number representing pH of this solution, P(10)=diammonium hydrogen phosphate at pH 10, Am = NH₄OH and S = NaOH.

Sample Codes	Eggshell solution (E = Ca source)		Diammonium hydrogen phosphate solution (P = P source)
	pH	Reagent for pH control	pH
E(5)Am-P(10)S	5	NH ₄ OH	10 (adjusted by using NaOH)
E(8)Am- P(10)S	8		
E(10)Am- P(10)S	10		
E(5)S- P(10)S	5	NaOH	
E(8)S- P(10)S	8		
E(10)S- P(10)S	10		

Table 3.4: Samples were synthesised via hydrothermal synthesis process at 350 °C from eggshell solutions of pH 5 and 10 and diammonium hydrogen phosphate solution of pH 8 and 10, where pHs of both solutions were adjusted using NH₄OH and NaOH.

Sample Codes	Eggshell solution (E = Ca source)		Diammonium hydrogen phosphate solution (P = P source)	
	pH	Reagent for pH control	pH	Reagent for pH control
E(5)Am-P(10)Am 350	5	NH ₄ OH	10	NH ₄ OH
E(10)Am-P(8)Am 350	10		8	
E(10)Am-P(10)Am 350	10		10	
E(10)S-P(10)S 350	10	NaOH	10	NaOH

3.2.1.2.5.2 Sample preparation using Ca(NO₃)₂·4H₂O as Ca and struvite as P sources

Five samples were further prepared at 200 and 350 °C using *struvite solution with Ca(NO₃)₂·4H₂O* are presented in Table 3.5 following the same procedures of solution flow rate and reactor pressure as mentioned above.

Table 3.5: Five samples were synthesised via hydrothermal synthesis process at 200 and 350 °C at pH (10 - 11) of calcium nitrate tetra hydrate $[Ca(NO_3)_2 \cdot 4H_2O]$ solution and at a constant pH 6 of struvite solution. Where pH of both solutions was adjusted using NH_4OH and $NaOH$. $Ca(W-Am)$ = calcium nitrate tetra hydrate with corresponding label representing dissolution media (W for DI water) of precursor powder and pH buffer, St=Struvite at pH 6, N = HNO_3 , Am = NH_4OH , S = $NaOH$.

Sample Codes	Reaction temperature (°C)	Calcium nitrate solution (Ca = Ca source)			Struvite solution (St = P source)				
		pH	Dissolution media	Reagent for pH control	pH	Dissolution media	Reagent for pH control		
Ca(W-Am)-St(N-Am) 200	200	10-11	De-ionised water	NH ₄ OH	6	HNO ₃	NH ₄ OH		
Ca(W-S)-St(N-S) 200				NaOH			NaOH		
Ca(W-Am)-St(Ac-Am) 200				NH ₄ OH		CH ₃ COOH	NH ₄ OH		
Ca(W-Am)-St(N-Am) 350	350							HNO ₃	
Ca(W-Am)-St(Ac-Am) 350								CH ₃ COOH	

3.2.1.2.5.3 Sample preparation using eggshells as Ca and struvite as P sources

Finally, five samples were prepared at 200 and 350 °C using *struvite solution and eggshell solution* are presented in Table 3.6 following the same procedures of solution flow rate and reactor pressure as mentioned above.

Table 3.6: Samples were synthesised via hydrothermal synthesis process at 200 and 350 °C at pH (10 - 11) of eggshell solution and at a constant pH 6 of struvite solution. Where pH of both solutions was adjusted using NH_4OH and NaOH . E(N/Ac-Am) = Eggshells with corresponding label representing dissolution media of precursor powder and pH buffer, St=Struvite at pH 6, N = HNO_3 , Ac = CH_3COOH , Am = NH_4OH , S = NaOH .

Sample Codes	Reaction temperature (°C)	Eggshell solution (E = Ca source)			Struvite solution (St = P source)		
		pH	Acid to dissolve Ca precursor	Reagent for pH control	pH	Acid to dissolve struvite	Reagent for pH control
E(N-Am)-St(N-Am) 200	200	10-11	HNO ₃	NH ₄ OH	6	HNO ₃	NH ₄ OH
E(N-S)-St(N-S) 200				NaOH			NaOH
E(Ac-Am)-St(Ac-Am) 200			CH ₃ COOH	NH ₄ OH	CH ₃ COOH	NH ₄ OH	
E(N-Am)-St(N-Am) 350	350		HNO ₃		HNO ₃		
E(Ac-Am)-St(Ac-Am) 350			CH ₃ COOH		CH ₃ COOH		

3.2.1.3 Ball milling followed by sintering method

The pre-treated eggshells obtained by following the several steps mentioned above named as raw eggshells (E) and pre-treated eggshells were also calcined at 700 and 900 °C at 10 °C /min for 2 hr, named as calcined eggshells (Ecal).

The procedure of CaPs preparation using raw eggshells and/or calcined eggshells (at 900 °C) and struvite via ball milling followed by sintering is presented in Figure 3.4 below. The precursor materials were mixed at a Ca/P ratio of 1.67.

De-ionised (DI) water was added (precursors: DI Water = 1.2 : 1 (w/v)) to the precursor materials to get homogeneous mixture and then wet-milled them in a planetary ball-mill (PM-100, Retsch, Germany) for 2 hrs at a speed of 400 rpm in a zirconia bowl. Ball milled samples were then dried overnight at 50 °C, ground and sieved to obtain particle size below 45 µm and then sintered at sintered at 700, 900 and 1150 °C for 3 hr at 5 °C/min ramp.

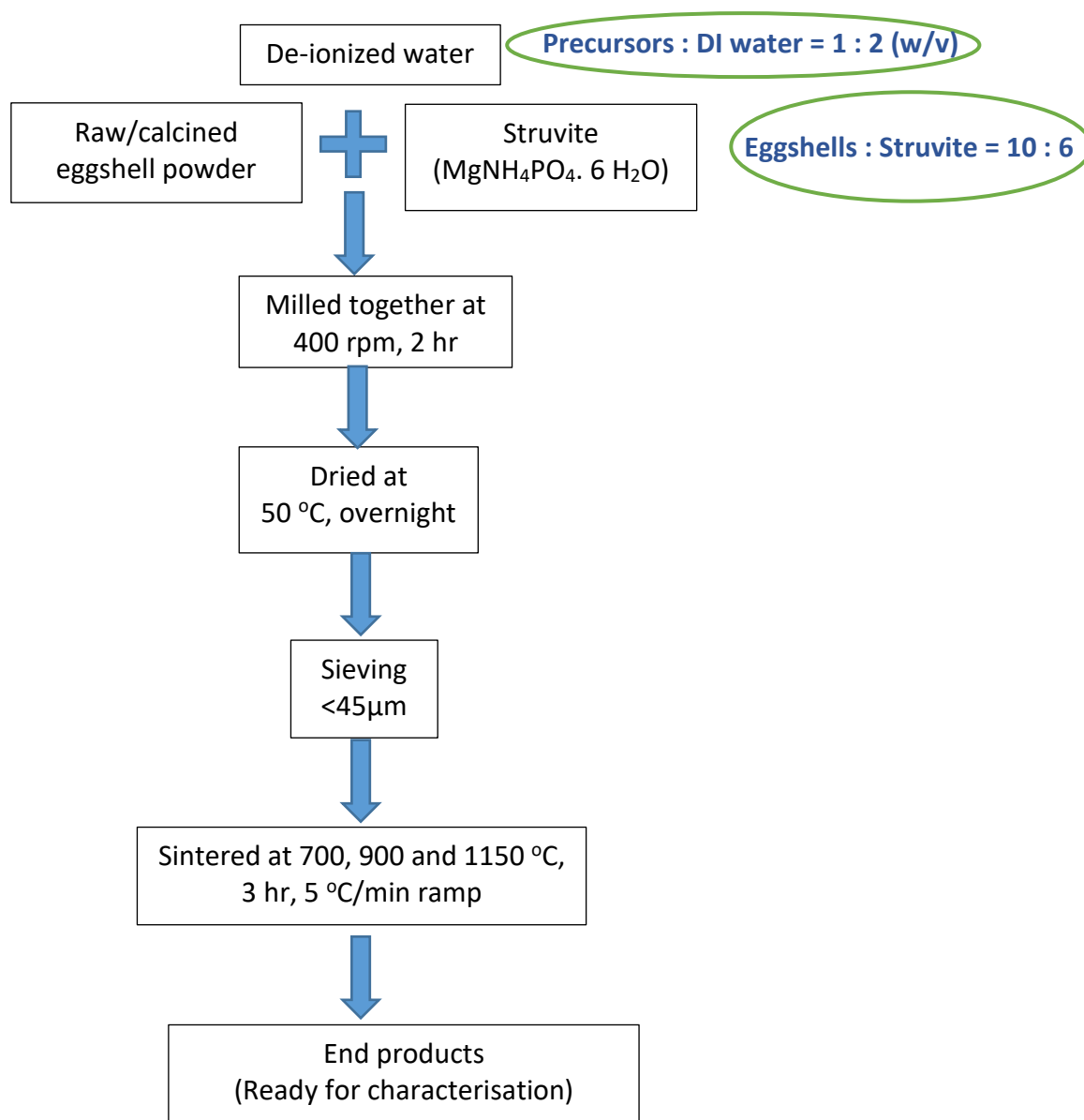


Figure 3.4: Proposed CaPs synthesis protocol via ball milling followed by sintering.

3.2.1.4 Manufacturing of Microspheres via flame-spheroidisation method

The ball milled samples prepared using same protocol as mentioned above were taken to prepare microspheres via flame spheroidisation. As the dried particles obtained after ball milling were agglomerated together, particles were ground gently using mortar and pestle and sieved to obtain granules of two different size ranges of 63 – 100 µm and 100 -160 µm and then spheroidised using oxygen/acetylene gas flame using 3:3 gas flow rate ratio. The

protocol of preparation of CaPs microspheres from eggshells and struvite is given below in Figure 3.5.

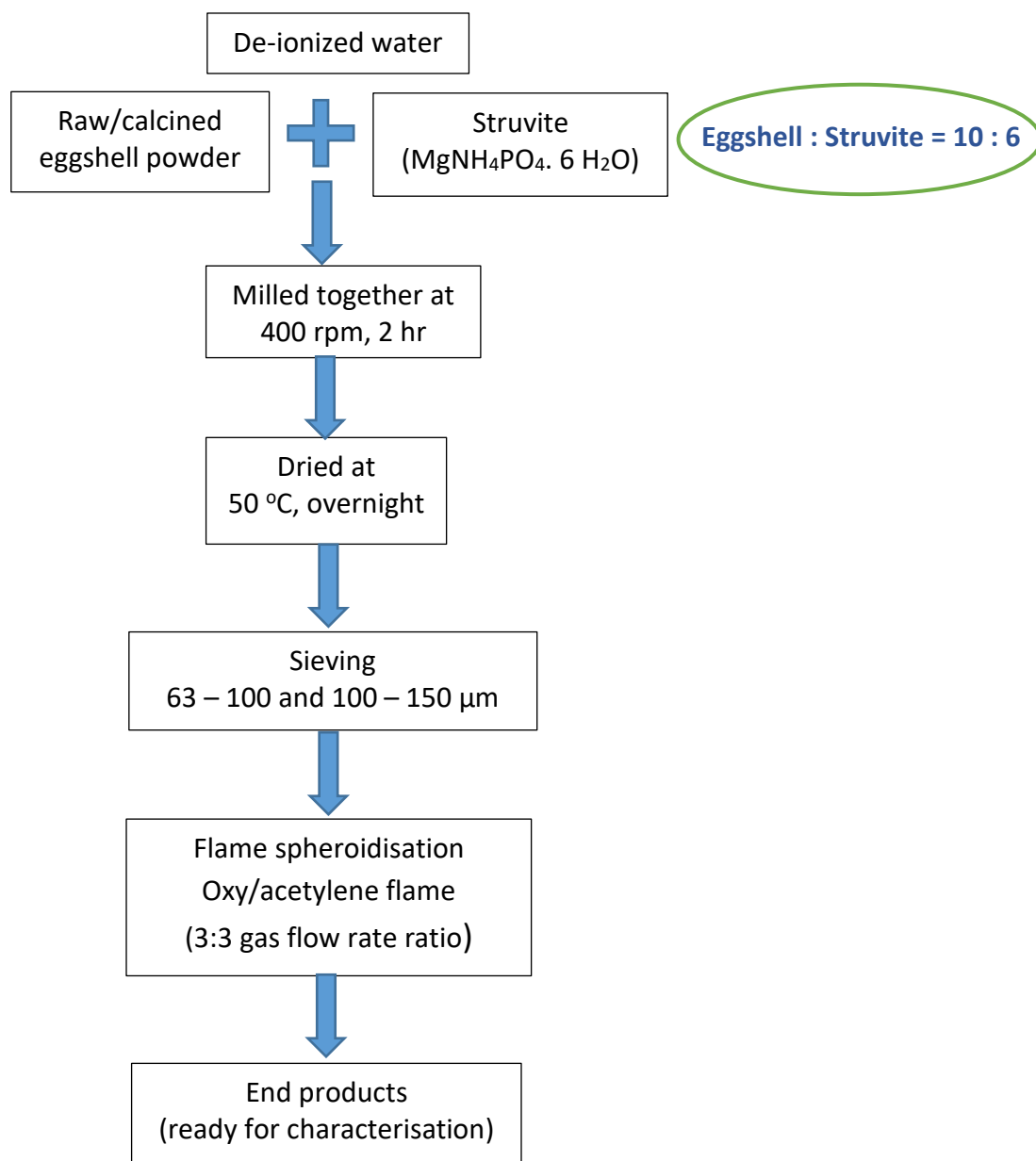


Figure 3.5: Proposed CaPs microspheres synthesis protocol via ball milling followed by flame spheroidisation.

3.2.1.5 Fabrication of composites (scaffolds and films) using Eggshells-Struvite and chitosan via Resonance Acoustic Mixing (RAM)

Bioceramic-polymer composites in the form of scaffolds and films were fabricated from the CaPs obtained via hydrothermal synthesis [i.e. E(Ac-Am)-St(Ac-Am)350], ball milling followed by sintering [i.e. Ecal-St(bm) <25 μm] and flame spheroidisation [i.e. Ecal-St(fs)63-100 microspheres] (Table 3.7) and commercially available chitosan (Ch) via resonance acoustic mixing method using Resodyn acoustic mixers (LabRAM I) (Figure 3.6). Commercially available HA (Promethean Particles Ltd.) and Ch were also utilised to prepare composites (scaffolds and films) as a control using the same method. 2% Ch solution was prepared in 2% acetic acid and HA/CaPs suspensions in DI water with the ratio of 1:50 (w/v) were prepared prior to composite fabrication using LabRAM I. The 50:50 (w/w) ratio of HA/CaPs and Ch was maintained for the preparation of the composites. Mixing acceleration and time were different for different batches to obtain a homogeneous mixture. However, the suspension of microspheres was not prepared due to the bigger particle size ranges of microspheres which could be settled down quickly compared to the other CaPs obtained. The required mixing acceleration and time for the preparation of HA/CaPs suspensions and composite suspensions (HA/CaPs-Ch) of different batches are presented in the Table 3.8 and Table 3.9, respectively.

Table 3.7: The sample codes of the composites fabricated via Resonance Acoustic Mixing (RAM) from the commercial chitosan (Ch) mixing with commercial HA (HA_{com}) or calcium phosphates (CaPs) obtained (including respective phases) in this study via hydrothermal synthesis (CaP_{ht}), ball milling followed by sintering (CaP_{bm}), followed by flame spheroidisation (CaP_{fs}).

Sample codes	HA/CaPs samples used		Phases of CaPs samples
	Methods	Selected samples	
$\text{HA}_{\text{com}}\text{-Ch}$	Hydrothermal synthesis (Commercial)	HA_{com} (Promethean particles Ltd.)	Nano hydroxyapatite (nHA)
$\text{CaP}_{\text{ht}}\text{-Ch}$	Hydrothermal synthesis	CaP_{ht} : E(Ac-Am)-St(Ac-Am) 350	Mg-WH, HA
$\text{CaP}_{\text{bm}}\text{-Ch}$	Ball milling followed by sintering	CaP_{bm} : Ecal-St(bm)0 (<25 μm)	HA, Brucite (Mg(OH) ₂)
$\text{CaP}_{\text{fs}}\text{-Ch}$	Flame spheroidisation	CaP_{fs} : Ecal-St(fs) (63-100)	MgO, α -TCP, HA

Table 3.8: The required mixing acceleration and time to prepare different batches of HA/CaP suspensions.

HA/CaP suspensions	Mixing acceleration (g)	Mixing time (min)	Number of repeat samples
HA _{com}	30	0.5	1
	50	2	
	70	5	
	80	10	
	30	0.5	1
	50	0.5	
	90	2	
CaP _{ht}	30	0.5	2
	50	2	
	70	5	
	90	5	
	50	2	1
	90	5	
	100	5	
CaP _{bm}	30	0.5	2
	50	5	
	90	10	
	50	1	1
	100	10	
CaP _{fs}	-		

Table 3.9: The required mixing acceleration and time to prepare different HA/CaPs-Ch composite suspensions.

Samples name	Mixing acceleration (g)	Mixing time (min)	Repetition
HA _{com} -Ch	30	0.5	1
	50	2	
	70	5	
	80	10	
CaP _{ht} -Ch	30	1	2
	50	2	
	70	5	
	90	10	
CaP _{bm} -Ch	30	0.5	2
	50	5	
	90	10	
	100	10	1
CaP _{fs} -Ch	30	3	3
	50	5	
	70	5	
	90	5	
	50	2	6
	90	3	
	100	5	

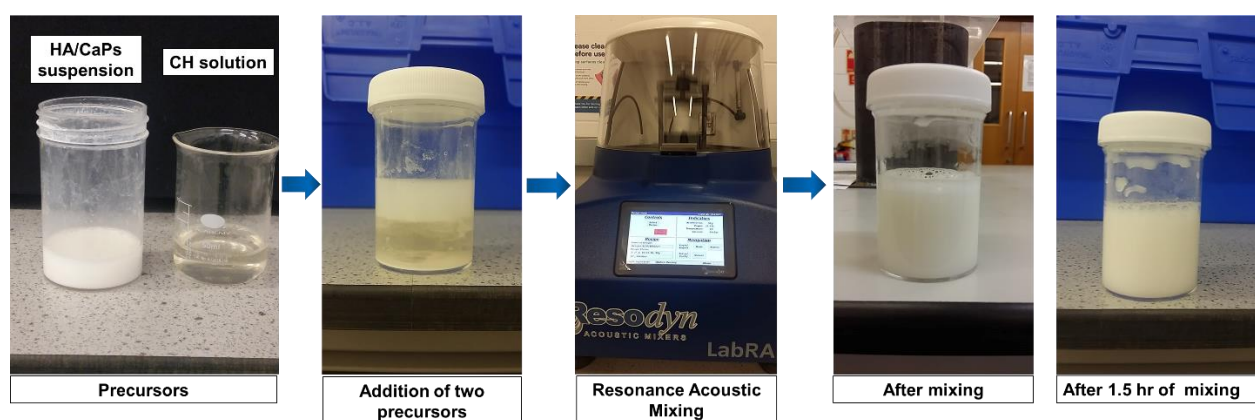


Figure 3.6: The preparation of HA/CaPs-Ch composite suspensions via resonance acoustic mixing (RAM) method.

The composite suspensions were left overnight to remove the bubbles. However, a vacuum oven was used for quick removal of the bubbles only for the composite suspension containing microspheres and cast in the mould quickly to avoid settling down the microspheres.

A 30 ml of each composite suspension was cast on a petri-dish using a syringe to prepare a film and left under a fume hood for drying.

To fabricate scaffolds, a 20 ml of each composite suspension was cast in PTFE (Polytetrafluoroethylene) mould (10×10), freezed at -100 °C and the frozen structure was then transferred to a freeze dryer (Coolsafe 100-9 Pro) and left for at least 48 h to fully dehydrate.

3.2.1.5.1 Crosslinking of the scaffolds

To explore the effect of crosslinker on the mechanical properties of the scaffolds, 1% Genipin solution was prepared using ethanol. Three replicates from each set of scaffolds were soaked into 30 ml of Genipin solution and the vials were sealed with paraffin film to avoid ethanol evaporation during crosslinking process. The sealed vials were then kept in the oven (37 °C) for 24 hrs and then the scaffolds were safely removed from the vials using a tweezer, washed thoroughly with de-ionised water and dried in a freeze dryer.

3.2.1.5.2 Swelling properties and weight loss of scaffolds

Swelling properties of the scaffolds and the films were carried out in PBS solution. Samples were first dried in the oven at 50 °C and the weight of each sample was measured (W_0). Each sample was then submerged into the 10 ml PBS solution (pH=7.4) in a vial at 37 °C. The experiment was done in three replicates of each set. After 15 min the samples were picked from the solution, dried using filter paper, and weighed (W_f). They were then put back into the solution to continue the process for the next 30 min, 1 hr, then every hr till 6 hrs, 24 hrs, 26hrs, 28 hrs and 30 hrs.

After completing the swelling properties testing, samples were dried overnight at 50 °C and weighed (W_f) to measure the weight loss of each sample.

3.3 Characterisation techniques

3.3.1 Elemental analysis via ICP-MS

Compositional analysis of the eggshell powder was conducted using inductively coupled plasma mass spectroscopy (ICP-MS, Thermo-Fisher Scientific iCAP-Q equipped with collision cell technology with energy discrimination) to verify their composition. 0.1 g of eggshells was digested in 50 ml 37% HCl until a clear solution was obtained. The solution was then 50% diluted with Milli-Q water (1:1). The resultant solution was then diluted with 2% HNO₃ with 1:10 ratio. Then the final solution was filtered through a 0.2µm syringe filter for ICPMS analysis. Moreover, blank samples of pure Milli-Q water were mixed with the same proportion of HCl and 2% HNO₃ to be used as a standard calibration solution. Three replicates were analysed, and the average values reported.

3.3.2 X-ray diffraction (XRD)

X-ray diffraction studies were conducted to confirm the phase identification of the samples produced using a Bruker D8 Advance with DaVinci diffractometer. The instrument was operated at room temperature and ambient atmosphere with Ni-filtered CuKα radiation ($\lambda=0.15418$ nm), generated at 40 kV and 35 mA. Scans were performed with a step size of 0.01° and step time of 2 s over an angular range 2θ from 5° to 65°. Diffraction patterns were analysed using EVA processing software for phase identification.

The average crystallite size of the synthesised particles was also calculated by using the Scherrer's formula as follows in Equation 3 [94]. The instrumental broadening was not considered.

$$X_s = \frac{0.9\lambda}{FWHM \cos\theta} \quad \text{Equation 3}$$

where:

X_s = The average crystallite size (nm)

λ = The wavelength of the X-ray

FWHM = The full width at half maximum for the diffraction peak under consideration (rad)

θ (degree) = The Bragg angle.

Percentage of phases produced in biphasic CaPs were measured according to Equation 4 [230].

$$\text{Percentage of HA} = \frac{I_{\text{HA}(211)}}{I_{\text{HA}(211)} + I_{\text{Mg-WH}(0210)}} \quad \text{Equation 4}$$

where,

$I_{\text{HA}(0210)}$ = Intensity of HA at (211)

$I_{\text{Mg-WH}(0210)}$ = Intensity of Mg-WH at (0210)

Crystallinity (%) values of the samples were obtained from the EVA software using automatic back ground correction.

3.3.3 Scanning electron microscopy (SEM) and energy dispersive X-ray spectrometer (EDX)

Sample morphology was examined using scanning electron microscopy (SEM, Philips (FEI) XL30, USA) and Field Emission Gun Scanning Electron Microscopy (JEOL 7100F FEG-SEM) whilst energy dispersive X-ray spectrometer (EDX) was used to determine the composition. Accelerating voltage of 5 kV was used for morphology analysis of the samples obtained via hydrothermal synthesis, whereas 15 kV was used for the ball milled samples, microspheres, and composites. EDX was conducted using an Oxford Instruments INCA EDX system with a Si-Li crystal detector and Oxford Instruments AZtec Energy Advanced X-max 150 EDS System for the chemical characterisation. Working distance of 10 mm was maintained for both SEM and EDX analysis. Samples were coated with iridium (Ir) using Quorum Iridium coater and platinum using Polaron Platinum Sputter Coaters (SC7640, UK) for acquiring good quality images via FEG-SEM and XL30 respectively. Carbon coating was done using Quorum Q150 for elemental analysis and EDX spectroscopy was conducted using working distance of 10 mm and a beam voltage of 15 kV. At least two different images of each sample and three different areas of each image were used to conduct elemental analysis.

3.3.4 Transmission electron microscopy (TEM)

TEM was conducted using a high-resolution TEM JEOL 2000fx at an accelerating voltage of 200 kV. The products in powder physical form were suspended in distilled water and dropped onto carbon coated copper grids (300 lines per inch) prior to drying under vacuum for 15 min.

3.3.5 Particle size analysis

Particle size analysis was carried out using ImageJ software from the FEG-SEM images. A range of 10-70 particles of each sample were selected to measure particle size depending on the morphology of particles. Width/ diameter for the platelet, rod and ellipsoidal morphology, inner diameter including thickness for the tube morphology were measured. However, both length and width for the rhombohedral shaped particles were measured.

3.3.6 FTIR analysis

Infrared spectroscopy was performed using a Bruker Tensor-27 spectrometer (Germany). All samples were scanned in transmittance mode in the region of 4000 to 550 cm^{-1} (wave numbers) and the spectra were collected with a resolution of 4 cm^{-1} by averaging 32 scans using a standard pike attenuated total reflectance (ATR) cell (Pike technology, UK). All spectra obtained were analysed using OpusTM software version 5.5.

3.3.7 Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis of prepared samples were carried out using a DSC instrument (TA Instruments SDT Q600, UK). Around 14 mg of samples were heated from room temperature to desired temperature (1200 °C) at 10 °C min^{-1} rate under flowing argon gas. A blank was also run in order to determine the baseline which was then subtracted from the traces obtained using TA universal analysis software.

3.3.8 Mechanical Testing

Compression testing of the scaffolds were carried out to measure the mechanical properties using Instron universal testing machine. A cross-head speed of 0.5 mm/min and a 100 N load cell were used. Tests were performed in triplicate.

Chapter 4

Manufacturing of calcium phosphates (CaPs) using waste resources via continuous flow hydrothermal synthesis method

4.1 Introduction

Calcium phosphates (CaPs) are the main mineral constituent of human bones and teeth and possess excellent biocompatibility, osteoconductive properties, nontoxicity, and chemical similarity to the inorganic components of the natural bone [231]. Therefore, the CaPs-containing biomaterials are suitable for bone regeneration and replacement in tissue engineering as well as other applications, like drug delivery agents, prosthetic coatings, and gene carriers [232, 233]. Among the CaPs family compounds, hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ (HA) is the most extensively used in the field of regenerative medicine for developing bone substitutes due to its chemical and physical resemblance to the mineral part of the bone tissue [150]. Human bone is a natural composite of nano-apatite rods (which are ,100 nm) and collagen. The biological HA is a nonstoichiometric, carbonated, and calcium-deficient form of apatite ($\text{Ca/P} < 1.67$), containing various amounts of positively charged ions (e.g., Mg^{2+} , Na^+ and K^+) and negatively charged ions (e.g., CO_3^{2-} , Cl^- and F^-), in substitution of Ca^{2+} or $\text{PO}_4^{3-}/\text{OH}^-$ ions, respectively [231]. Hence, it is of great interest to consider HA and other calcium phosphates (calcium deficient hydroxyapatite, CDHA) as components in injectable bone cements and as a biocompatible phase reinforcement in biomedical composites, for coatings on metal implants or filling bulk bone defect. However, the low mechanical strength of normal HAp ceramics generally restricts its use to low load-bearing applications [26]

The nano-hydroxyapatite (nHA) exhibits improved densification which enhances its mechanical properties including fracture toughness. Moreover, nHA as an implant material has shown better bioactivity and improved biocompatibility over coarser HA-based implants [26]. The nHA can be prepared via different methods such as coprecipitation, sol-gel synthesis, mechanical milling, hydrothermal reaction, etc. [231]. Among them, the hydrothermal synthesis process is an attractive technique for the processing of nanostructured materials. [231] A variety of hydrothermal methods have been reported in

literature regarding the production of nanostructured HA with a wide range of dimension and variety of morphological features, for example rods, needles, wires, whiskers fibres, sheets, plates, and organized rod spheres or sheet spheres etc. [231]. In comparison to other hydrothermal methods, continuous hydrothermal synthesis is green, cheap and relatively straightforward method that provides a highly scalable route for the preparation of inorganic nanomaterials [8, 9]. This process utilises the chemical and physical properties of superheated water to produce nanoparticles by rapid nucleation and precipitation within a continuous reactor. [229, 234].

Literature showed that in hydrothermal condition, HA nanoparticles with specific size, morphology, and aggregation of the crystals can be tailored in a closed system by varying the process parameters, such as nature of precursors and their concentration, saturation, temperature, pH, process time and the presence of potential additives [231].

Recently several studies have been carried out starting from various biogenic sources, such as marine algae, shells, corals, or wood to fabricate HA-based bone graft substitutes [90, 95, 231, 235, 236] with appropriate nanostructural characteristics closer to the living bone. Eggshells has widely been used in the biomaterial-based research as Ca sources to synthesise nHA or CaPs [235, 237], however some common synthetic chemicals e.g., diammonium phosphate $((\text{NH}_4)_2\text{HPO}_4)$ and orthophosphoric acid (H_3PO_4) have been used as a phosphate precursor [103, 237]. Recently, researchers are interested for studying on waste-derived P sources (i.e., struvite from waste water) [98, 238], especially for agricultural application. In a recent study, synthetic urine had been chosen as a P source to investigate the synthesis of HA biomaterials entirely from biowastes [103]. To the best of the author's knowledge, no studies had been reported on struvite as a P source to produce HA or CaPs for the application in the biomedical field.

This chapter focuses on the hydrothermal approaches employed in the development of nHA and/or CaPs from sustainable waste resources such as eggshells and struvite. Therefore, the nHA was prepared via hydrothermal synthesis processing utilising eggshells as Ca source and struvite as P source. The materials produced had been characterised using XRD, SEM, EDX, FTIR and TEM analyses. The effect of pH, base solution and temperature on the prepared samples were also investigated.

4.1 Results

In this section, the outcomes of the samples produced via hydrothermal synthesis from three different combinations of precursor solutions were reported in 3 experimental parts. In the first two parts, one of the precursor materials derived from waste resources was introduced instead of using both conventional chemical precursors. In the third part, both precursors used were waste-derived. Three different experimental parts of the hydrothermal synthesis process including the process parameters (i.e. temperature and pressure), chemicals used to obtain the required precursor solutions and characterisation techniques are highlighted in the flowchart in Figure 4.1.

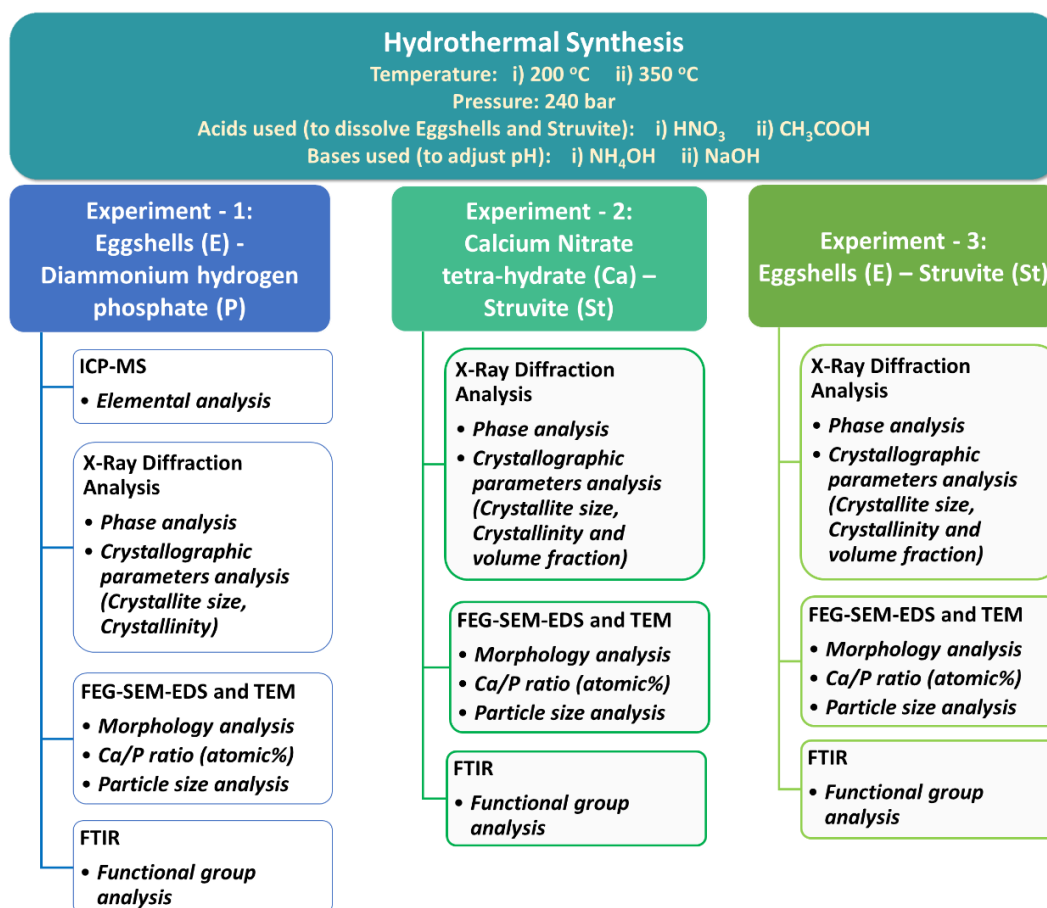


Figure 4.1: Schematic flow charts of experimental sections including process parameters and characterisation techniques used for the samples obtained via the hydrothermal synthesis process using conventional precursors (i.e. Di-ammonium hydrogen phosphate and Calcium nitrate tetra-hydrate) and natural waste resources (i.e. Eggshells and Struvite).

It is worth to be noted that the sample codes denoted using the symbols of precursors, acids, bases and pH mentioned below:

Experiment - 1: Eggshells (E) - Diammonium hydrogen phosphate (P)	Experiment - 2: Calcium Nitrate tetra-hydrate (Ca) - Struvite (St)	Experiment - 3: Eggshells (E) - Struvite (St)
• E(5/8/10)=eggshell with corresponding number representing pH of this solution • P(8/10)=diammonium hydrogen phosphate with corresponding number representing pH of this solution • Am = NH_4OH • S = NaOH	• Ca(W-Am/S) = calcium nitrate tetra hydrate with corresponding label representing dissolution media (W for DI water) and pH buffer (Am/S) • St(N/Ac-Am/S)= Struvite with corresponding label representing dissolution media (N/Ac) and pH buffer (Am/S) • N = HNO_3 • Ac = Acetic acid • Am = NH_4OH • S = NaOH	• E(N/Ac-Am/S) = Eggshells with corresponding label representing dissolution media (N/Ac) and pH buffer (Am/S) • St(N/Ac-Am/S)= Struvite with corresponding label representing dissolution media (N/Ac) and pH buffer (Am/S) • N = HNO_3 • Ac = CH_3COOH • Am = NH_4OH • S = NaOH

4.1.1 Hydroxyapatite synthesis using eggshell and di-ammonium hydrogen phosphate

4.1.1.1 Elemental analysis

Elemental analysis of the eggshells used was carried out via ICP-MS. Table 4.1 represents the mol% and wt% composition of the eggshells collected and it was found that a relative amount of 98 wt% of Ca was present with traces of other elements (see Table 4.1).

Table 4.1: Compositions of pre-treated eggshells via ICP-MS.

Components	Mol%	Wt%
Ca	96.38 ± 0.35	97.62 ± 0.43
Na	0.46 ± 0.08	0.27 ± 0.07
Mg	1.84 ± 0.01	1.13 ± 0.01
P	0.35 ± 0.07	0.27 ± 0.05
Sr	0.02 ± 0.01	0.04 ± 0.02
Zn	0.02 ± 0.01	0.03 ± 0.01
S	0.71 ± 0.09	0.58 ± 0.08
B	0.22 ± 0.08	0.06 ± 0.02

4.1.1.2 Investigating phase changes using XRD

Figure 4.2 shows the XRD pattern for the samples E(10)Am-P(10)Am 200 obtained from the initial trials of hydrothermal synthesis at 200 °C and 240 bar, where the eggshell solution was incorporated as the Ca (calcium) source and diammonium hydrogen phosphate solution as the P (phosphate) source. The pH of both solutions was maintained at 10 using NH₄OH. It was observed that apatite phase formed for both washed and unwashed samples and all of the reflections in the XRD patterns were comparable to the ICDD standard for hydroxyapatite (HA) (ICDD No. 00-009-0432). NH₄NO₃ peaks were observed along with HA peaks for the unwashed sample (i.e. as prepared) and after washing the materials with de-ionised water, no NH₄NO₃ peaks were detected. This highlighted that a wash step may be necessary to remove any residual NH₄NO₃ remaining from the synthesis process.

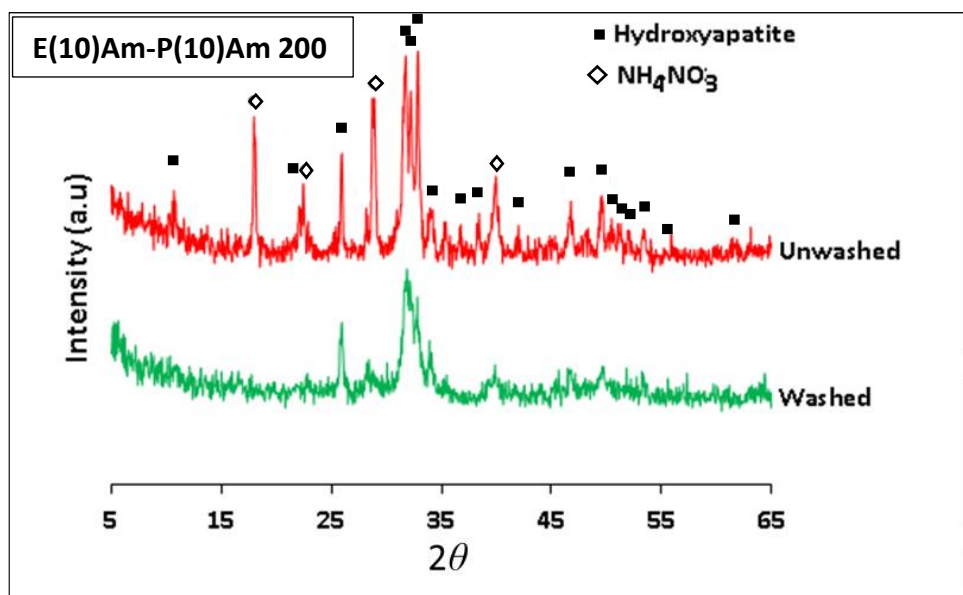


Figure 4.2: XRD pattern of washed and unwashed samples of E(10)Am-P(10)Am 200 produced from eggshell solution and diammonium hydrogen phosphate solution via the hydrothermal synthesis process at 200 °C and pH 10. The crystalline phases were indexed with hydroxyapatite (ICDD No. 00-009-0432) and NH_4NO_3 (ICDD No. 01-071-1760).

Figure 4.3A-C (below) represents the XRD pattern for three sets of experiments (six samples in each set) prepared at 200 °C using an eggshell solution of different pH (i.e. 5, 8 and 10 adjusted using NH_4OH and NaOH individually) and maintaining the diammonium hydrogen phosphate solution at constant pH 8 (i.e. for as prepared phosphate solution) (Figure 4.3A), pH 10 adjusted by NH_4OH (Figure 4.3B) and pH 10 adjusted by NaOH (Figure 4.3C). HA peaks were observed from all the combinations investigated. The crystalline phases identified were matched with hydroxyapatite (ICDD No. 00-009-0432).

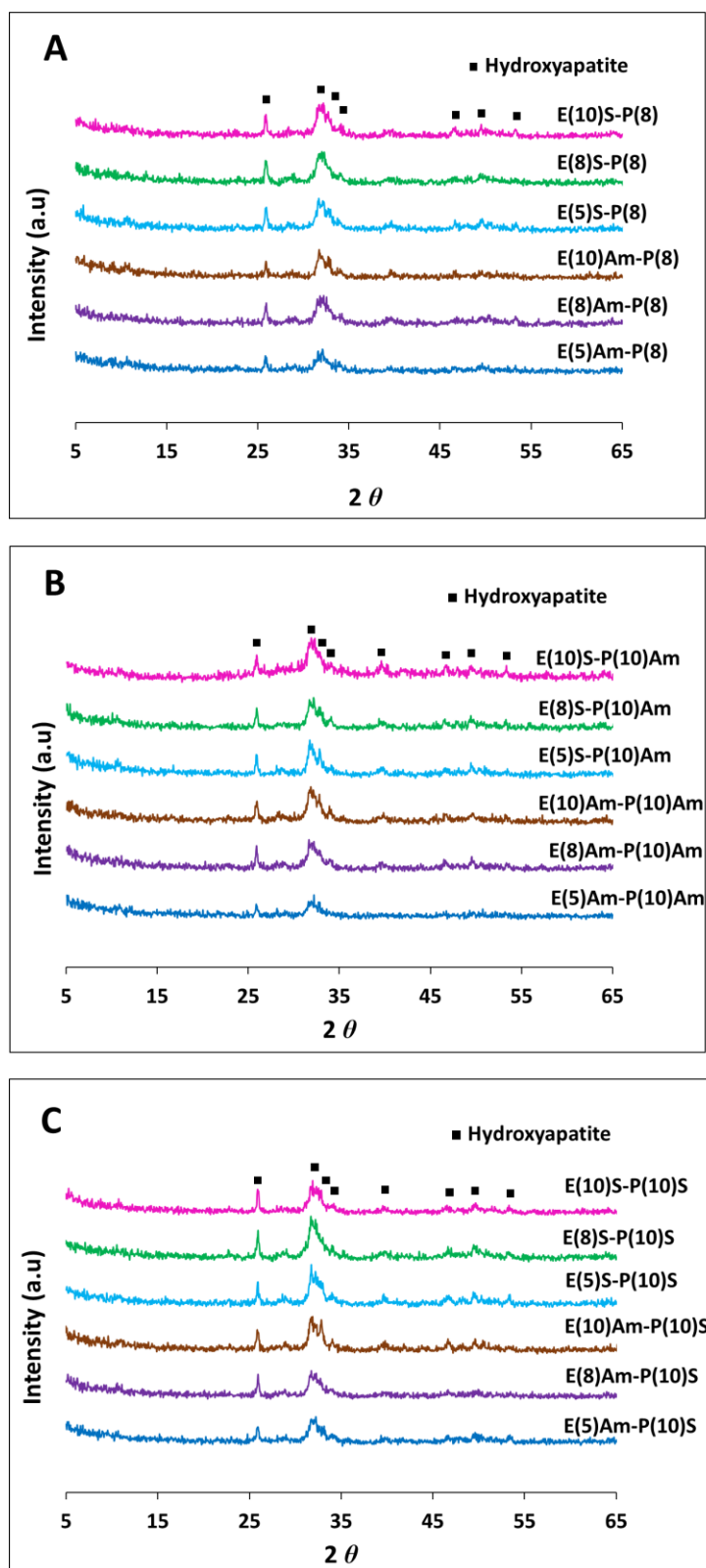


Figure 4.3: XRD pattern of samples synthesised at 200 °C via hydrothermal synthesis process at different pH (5, 8 and 10) of eggshells solution adjusted using NH_4OH and NaOH individually and at constant pH of diammonium phosphate solution: **(A)** pH 8 (as prepared), **(B)** pH 10 using NH_4OH and **(C)** pH 10 using NaOH .

To evaluate the effect of reaction temperature on the morphology of samples produced from eggshells solution and diammonium hydrogen phosphate solution, four further samples (i.e. E(10)Am-P(8), E(5)Am-P(10)Am, E(10)Am-P(10)Am and E(10)S-P(10)S) were also prepared at a higher temperature of 350 °C and compared with the same combinations produced at 200 °C. The XRD pattern of the samples produced from both sets using NH_4OH and NaOH to maintain the pH of precursors are shown in Figure 4.4A and 4B, respectively. HA peaks were confirmed for all combinations investigated by matching the crystalline phases with hydroxyapatite (ICDD No. 00-009-0432). However, more pronounced HA peaks were observed for the samples prepared at 350 °C compared to 200 °C.

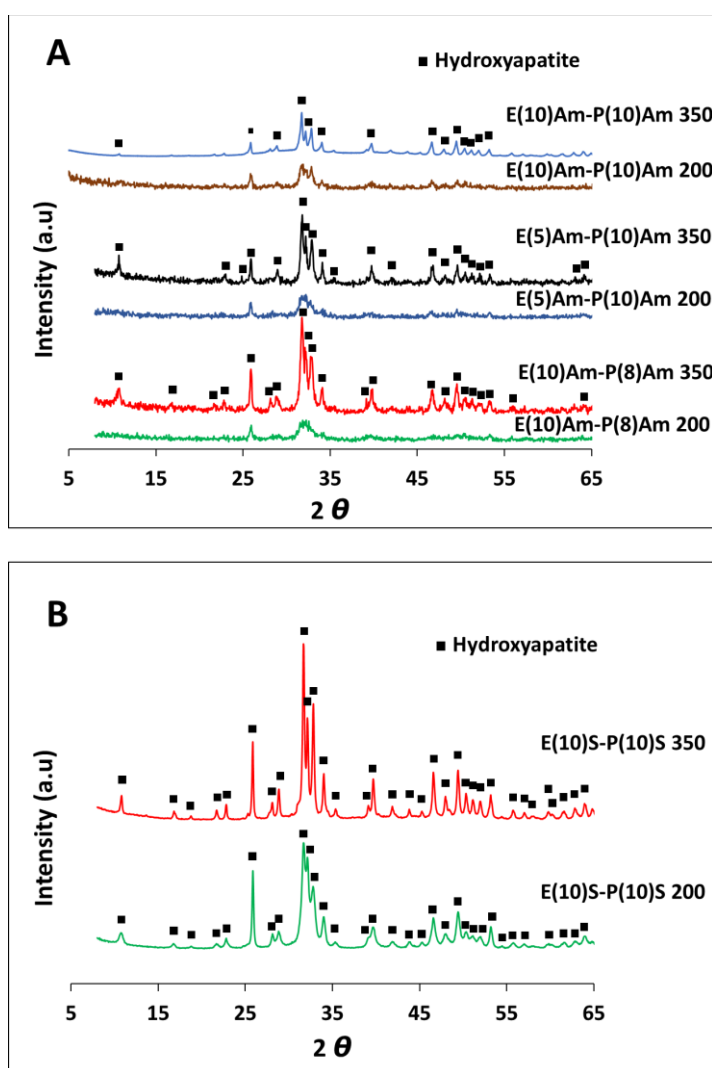


Figure 4.4: The XRD pattern of samples synthesised via hydrothermal synthesis at 350 °C and compared with samples produced at 200 °C at different pH of eggshells solution (i.e. pH 5 and 10) and diammonium hydrogen phosphate solution (i.e. pH 8 and 10) individually using (A) NH_4OH and (B) NaOH as a base solution to adjust pH.

4.1.1.3 Crystallite size and Crystallinity

Table 4.2 shows the crystallite size and percentage of crystallinity of the samples prepared at different pH of precursor solutions (i.e. pH 5, 8 and 10) and at different temperatures (i.e. 200 and 350 °C). The crystallite size of the samples responsible for Bragg reflections of the (002) plane was calculated from XRD data using the Scherrer equation [94]. Samples prepared at 200 °C were found to contain nanosized crystallites in the range of 30 - 80 nm. However, at a higher temperature of 350 °C, the crystallite size of samples was found to be 54 – 80 nm. At lower temperature of 200 °C, the percentage crystallinity of samples was 43 - 79%, whereas 65 - 80% of crystallinity was observed for samples processed at higher temperature (350 °C).

Table 4.2: Crystallite size at (002) plane and crystallinity of samples prepared from eggshell solution and diammonium hydrogen phosphate solution via hydrothermal process at 200 and 350 °C.

Samples	Reaction temperature (°C)	Crystallite size (nm) at Plane (002)	Crystallinity (%)
E(5)Am-P(8)	200	37	53
E(8)Am-P(8)		44	52
E(10)Am-P(8)		32	57
E(5)S-P(8)		40	53
E(8)S-P(8)		38	56
E(10)S-P(8)		68	56
E(5)Am-P(10)Am		69	60
E(8)Am- P(10)Am		50	59
E(10)Am- P(10)Am		52	64
E(5)S- P(10)Am		80	59
E(8)S- P(10)Am		59	57
E(10)S- P(10)Am		30	43
E(5)Am-P(10)S		33	58
E(8)Am- P(10)S		80	53
E(10)Am- P(10)S		54	60
E(5)S- P(10)S		65	54
E(8)S- P(10)S		62	56
E(10)S- P(10)S		58	79
E(10)Am-P(8) 350	350	54	69
E(5)Am-P(10)Am 350		69	65
E(10)Am- P(10)Am 350		65	69
E(10)S- P(10)S 350		80	80

4.1.1.4 Morphology characterisation of HA using FEG-SEM-EDS

Figure 4.5, Figure 4.6 and Figure 4.7 represent the surface morphology and Ca/P ratio of the eighteen hydrothermal synthesised samples prepared from eggshell solutions and diammonium hydrogen phosphate solutions at 200 °C. It was clear that all 18 samples prepared contained nano to submicron-sized particles, based on the 100 nm scale-bar portrayed in the images. Moreover, sheet-like morphology was clearly observed for all of the samples except E(10)Am-P(8), E(10)Am-P(10)Am and E(10)Am-P(10)S. The morphology of these three samples appears to be a mixture of platelets and rod-shaped particles. As similar morphology was seen for all these samples, E(10)Am-P(10)Am 200 and E(10)S-P(10)S 200 were selected for TEM analysis for further confirmation of crystal shapes and size.

The ratios of Ca/P obtained via EDX analysis for the 18 samples prepared varied from 1.43 for E(8)Am-P(8) to 1.63 for E(8)Am-P(10)S.

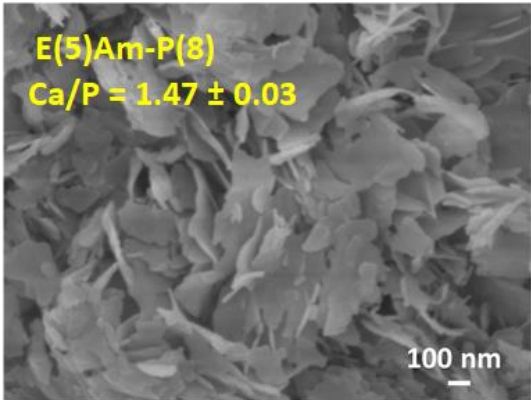
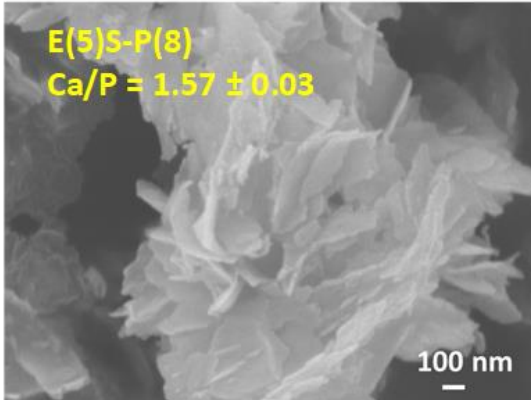
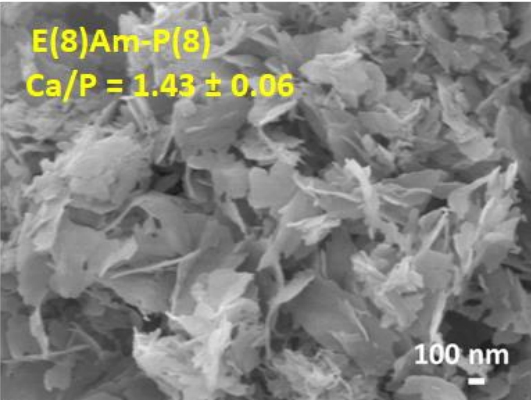
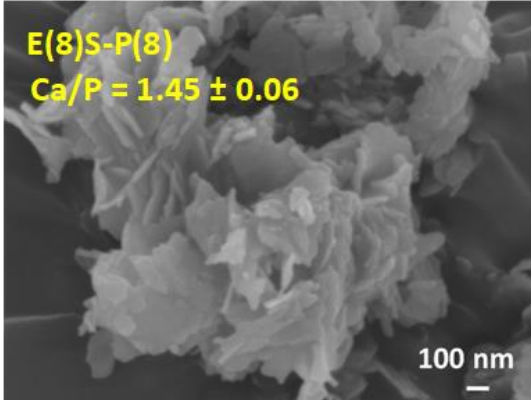
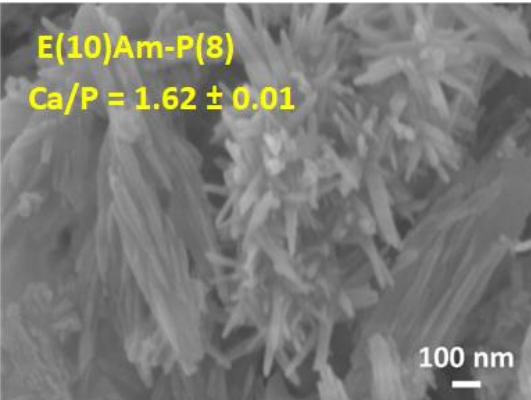
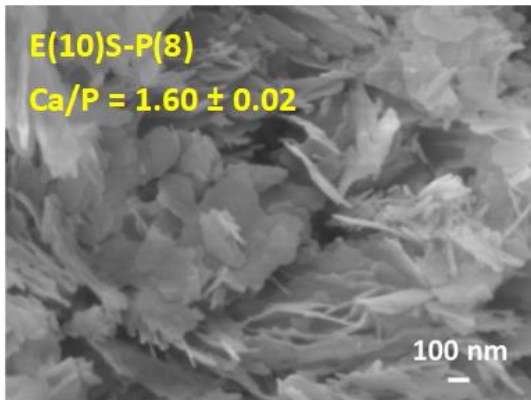
		<i>The base solution to adjust pH of eggshells solution</i>	
		<i>NH₄OH</i>	<i>NaOH</i>
<i>The pH of eggshells solution</i>	5	 E(5)Am-P(8) Ca/P = 1.47 ± 0.03	 E(5)S-P(8) Ca/P = 1.57 ± 0.03
	8	 E(8)Am-P(8) Ca/P = 1.43 ± 0.06	 E(8)S-P(8) Ca/P = 1.45 ± 0.06
	10	 E(10)Am-P(8) Ca/P = 1.62 ± 0.01	 E(10)S-P(8) Ca/P = 1.60 ± 0.02

Figure 4.5: Surface morphology and Ca/P ratio (atomic) of samples synthesised at 200 °C via hydrothermal process at different pH (5, 8 and 10) of eggshells solution adjusted by using NH₄OH and NaOH and at constant **pH 8 (as prepared) of diammonium phosphate solution.**

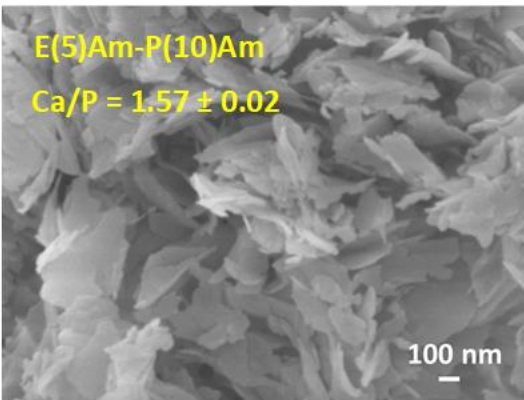
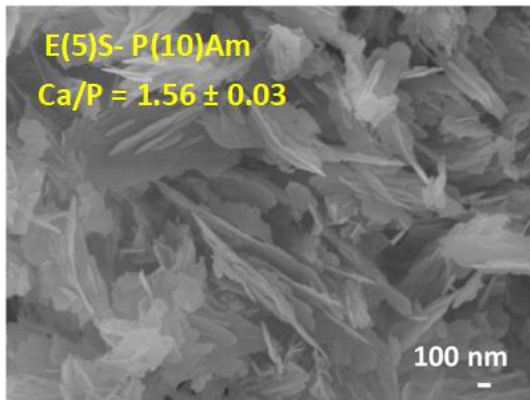
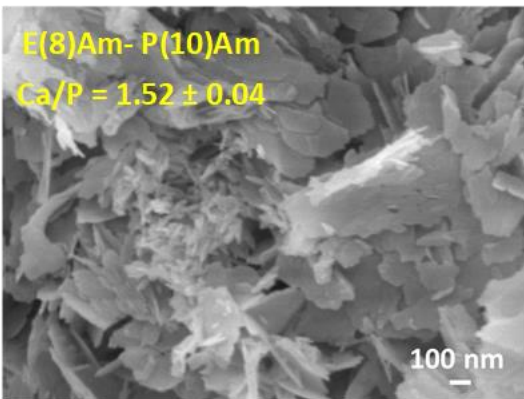
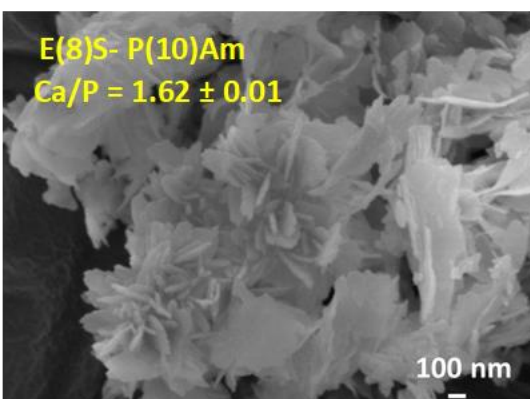
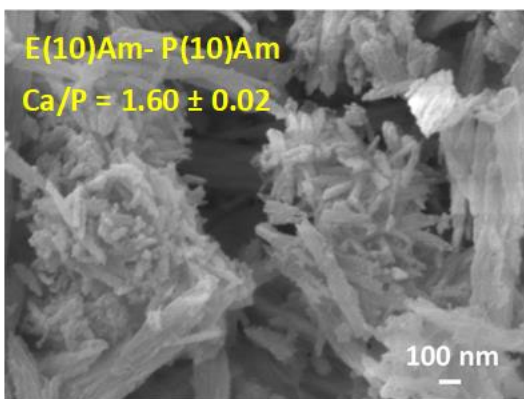
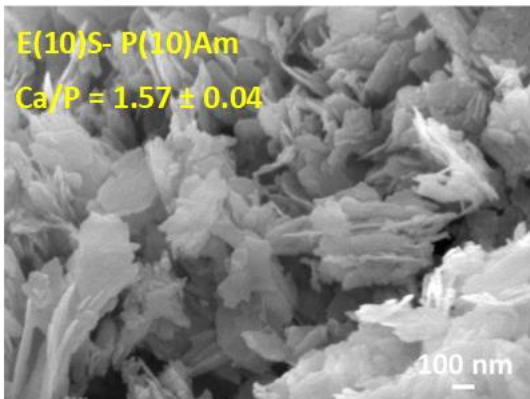
The pH the of eggshell solution		The base solution to adjust pH of the eggshell solution	
		NH_4OH	NaOH
	5		
	8		
	10		

Figure 4.6: Surface morphology and Ca/P ratio (atomic) of samples synthesised at 200 °C via hydrothermal process at different pH (5, 8 and 10) of eggshells solution adjusted using NH_4OH and NaOH and at **constant pH 10 of diammonium phosphate solution adjusted using NH_4OH .**

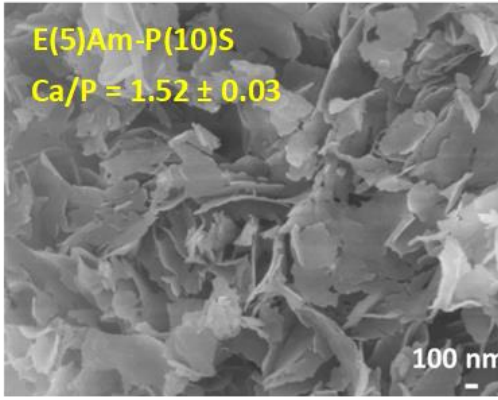
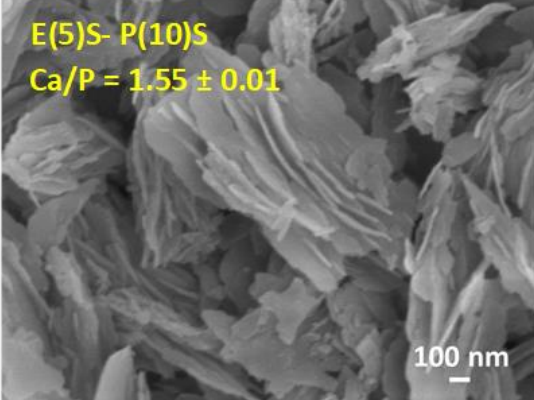
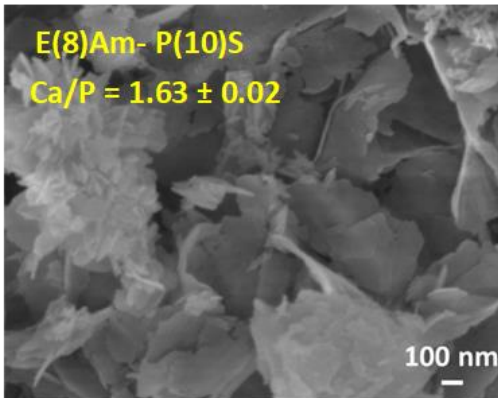
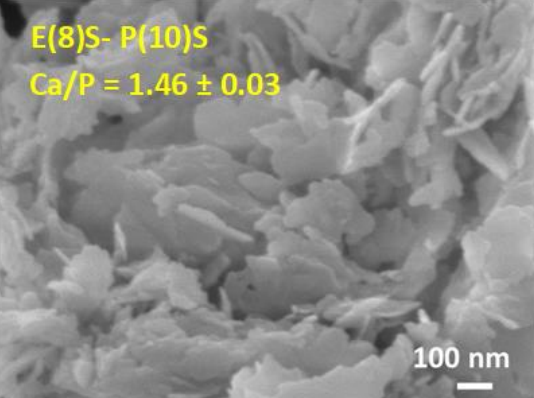
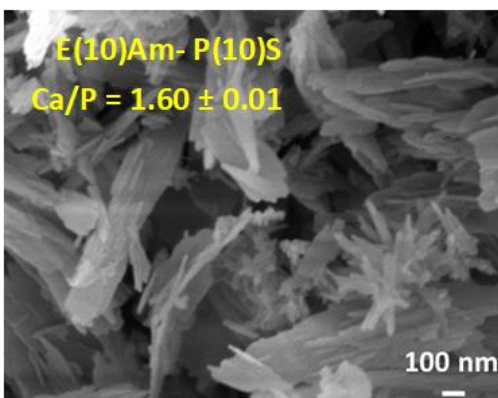
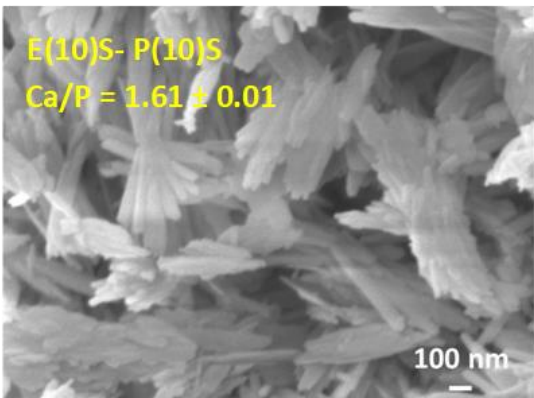
The pH of the eggshell solution		Base solution to adjust pH of eggshells solution	
		NH_4OH	NaOH
	5	 E(5)Am-P(10)S Ca/P = 1.52 ± 0.03	 E(5)S- P(10)S Ca/P = 1.55 ± 0.01
	8	 E(8)Am- P(10)S Ca/P = 1.63 ± 0.02	 E(8)S- P(10)S Ca/P = 1.46 ± 0.03
	10	 E(10)Am- P(10)S Ca/P = 1.60 ± 0.01	 E(10)S- P(10)S Ca/P = 1.61 ± 0.01

Figure 4.7: Surface morphology and Ca/P ratio (atomic) of samples synthesised at 200 °C via hydrothermal process at different pH (5, 8 and 10) of eggshells solution adjusted by using NH_4OH and NaOH and at **constant pH 10 of diammonium phosphate solution adjusted by using NaOH .**

A comparative study of surface morphology and Ca/P ratio (atomic %) of samples prepared at 200 °C and 350 °C via hydrothermal synthesis are shown in Figure 4.8. At higher temperature, mixed shapes were seen for E(5)Am-P(10)Am, platelet-shaped for E(10)Am-P(8)Am, tube shapes for E(10)Am-P(10)Am and rod shapes for E(10)S-P(10)S were observed.

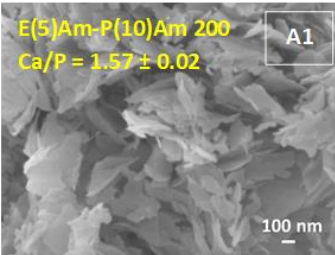
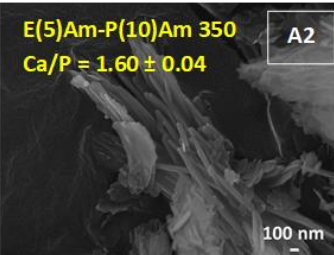
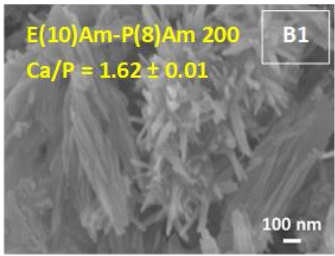
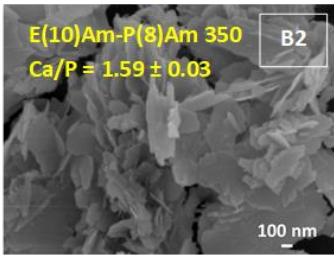
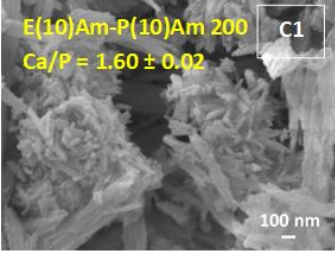
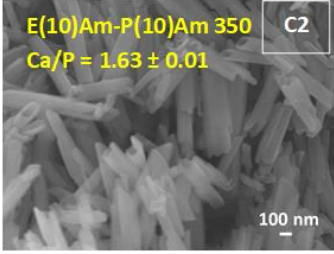
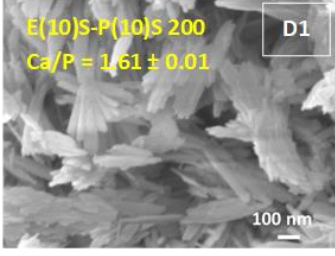
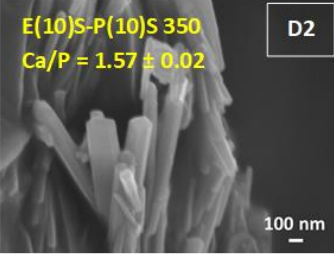
pH of the precursors		Base solution to adjust pH	Temperature	
Eggshells solution	Phosphate solution		200 °C	350 °C
5	10	NH ₄ OH	 E(5)Am-P(10)Am 200 Ca/P = 1.57 ± 0.02 A1	 E(5)Am-P(10)Am 350 Ca/P = 1.60 ± 0.04 A2
10	8		 E(10)Am-P(8)Am 200 Ca/P = 1.62 ± 0.01 B1	 E(10)Am-P(8)Am 350 Ca/P = 1.59 ± 0.03 B2
10	10		 E(10)Am-P(10)Am 200 Ca/P = 1.60 ± 0.02 C1	 E(10)Am-P(10)Am 350 Ca/P = 1.63 ± 0.01 C2
10	10	NaOH	 E(10)S-P(10)S 200 Ca/P = 1.61 ± 0.01 D1	 E(10)S-P(10)S 350 Ca/P = 1.57 ± 0.02 D2

Figure 4.8: Surface morphology and Ca/P ratio (atomic) of samples (E(5)Am-P(10)Am, E(10)Am-P(8)Am, E(10)Am-P(10)Am and E(10)S-P(10)S) synthesised using eggshells solution and diammonium hydrogen phosphate solution at 200 °C and 350 °C and at different pH adjusted using NH₄OH (A-C) and NaOH (D) via hydrothermal process.

4.1.1.5 Transmission Electron Microscopy

Particle morphology for samples E(10)Am-P(10)Am 200 and E(10)S-P(10)S 200 observed via TEM are presented in Figure 4.9. The particles highlighted in yellow circles for 3 different angles at different tilting positions (0 and ± 30 degree angle) (Figure 4.9A) clearly indicated platelet-like morphology for E(10)Am-P(10)Am 200. The morphology of E(10)S-P(10)S 200 was seemed to be platelet-shaped as well. However, the observed rod-shaped particles in the SEM image for the same sample was not clearly identified from the single TEM image (Figure 4.9B).

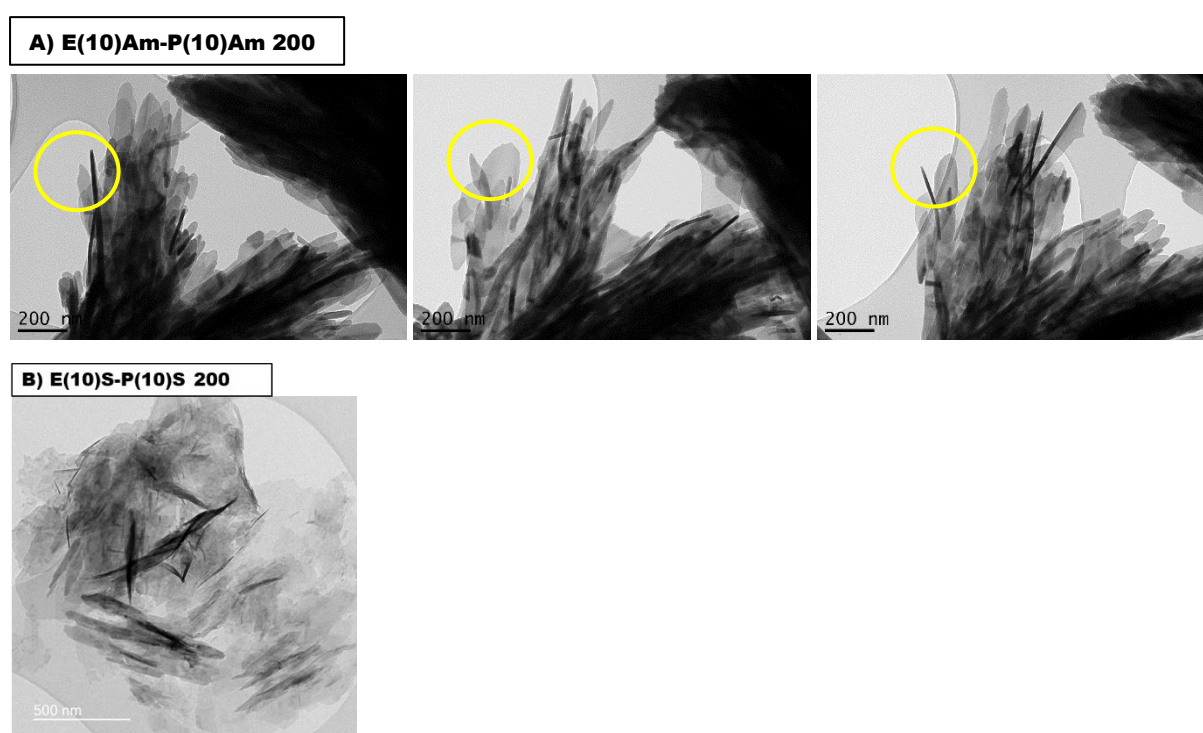


Figure 4.9: TEM images of sample A) E(10)Am-P(10)Am (at tilting positions; 0 and ± 30 degree angle) prepared at 200 °C from eggshells solution (pH 10) and diammonium hydrogen phosphate solution (pH 10) where NH_4OH was used to adjust pH for both solutions and B) E(10)S-P(10)S prepared at 200 °C and pH of both eggshells and diammonium hydrogen phosphate solution was maintained at 10 using NaOH.

Figure 4.10A and Figure 4.10B show TEM images for samples E(5)Am-P(10)Am 350 and E(10)Am-P(8)Am 350 which were prepared from eggshells solution (pH 5 and 10 respectively) and diammonium hydrogen phosphate solution (pH 10 and 8 respectively) at 350 °C. Particle shapes were again investigated by tilting the positions of the specimen at three different angles (0 and ± 30 degree angle). The particles highlighted in yellow circles for 3 different angles revealed rod-shaped morphology for E(5)Am-P(10)Am 350 with diameters of 10-70 nm (as measured by ImageJ) (see Figure 4.10A). However, mixed crystal shapes (i.e. platelets and rods) were identified for E(10)Am-P(8)Am sample with diameters of 15-110 nm (see Figure 4.10B; images b1-b3 shows platelet shape and images c1-c3 reveals rod shape). The tube shape for the sample E(10)Am-P(10)Am 350 and rod shape for E(10)S-P(10)S 350 were observed clearly in Figure 4.10C and D, respectively.

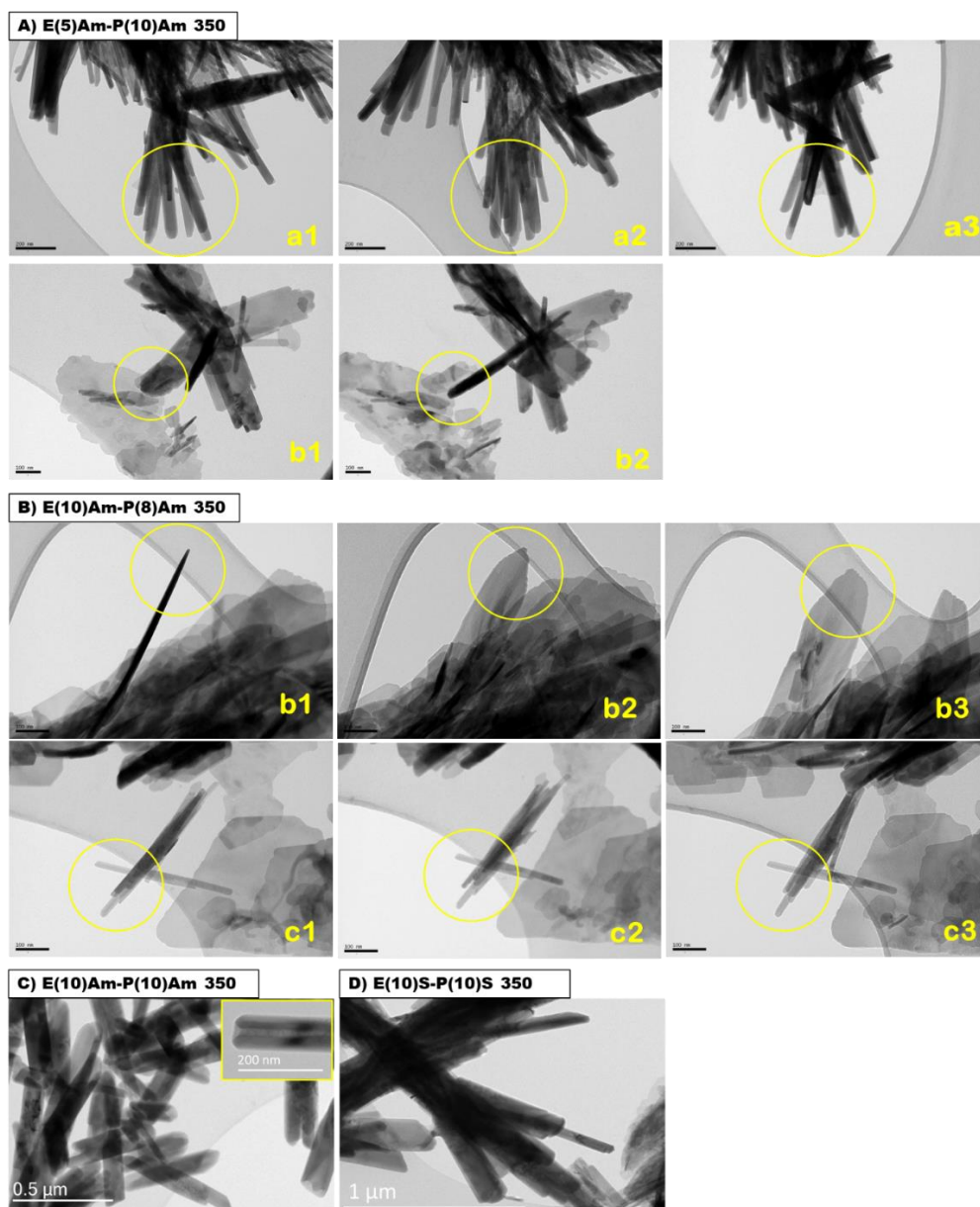


Figure 4.10: TEM images (at tilting positions; 0 and ± 30 degree angle) of samples prepared at 350 °C from (A) eggshells solution (pH 5) and diammonium hydrogen phosphate solution (pH 10) (E(5)Am-P(10)Am 350), (B) eggshells solution (pH 10) and diammonium hydrogen phosphate solution (pH 8) (E(10)Am-P(8)Am 350), (C) eggshells solution (pH 10) and diammonium hydrogen phosphate solution (pH 10) (E(10)Am-P(10)Am 350) and (D) eggshells solution (pH 10) and diammonium hydrogen phosphate solution (pH 8) (E(10)S-P(10)S 350). NH_4OH base solution was used to adjust the pH for both solutions of (A), (B) and (C), whereas NaOH for those of (D).

4.1.1.6 Particle Size analysis from FEG-SEM images

The particle size range of the samples produced was measured from FEG-SEM images via ImageJ software and presented in Table 4.3. At 200 °C, most of the samples produced showed sheet-like morphology of 37 – 331 nm. Rod-shaped particles were found in the range of 11 – 35 nm along with sheet-like particles for E(10)Am-P(8) and E(10)Am-P(10)S at the lower temperature. E(5)Am-P(10)Am 350 and E(10)Am-P(8) 350 also showed combinations of rod and sheet-like morphology at a higher temperature of 350 °C, where the size range of sheet and rod-shaped particles were found to be 37 – 340 nm and 21 – 86 nm, respectively. Interestingly, tube morphology (of 32 – 212 nm inner diameter and 15 – 49 nm of thickness) were observed for E(10)Am- P(10)Am 350 at 350 °C, whereas only rod morphology (of 32- 86 nm dia) was found for E(10)S- P(10)S at both temperatures. The particle size of six samples could not be measured due to the higher agglomeration of these particles.

Table 4.3: Particle size range of samples prepared from eggshell solution and diammonium hydrogen phosphate solution via hydrothermal process at 200 and 350 °C.

Samples name	Reaction temperature (°C)	Particle size range (nm)	Morphology
E(5)Am-P(8)	200	42- 312	Sheet
E(8)Am-P(8)		-	Sheet
E(10)Am-P(8)		43 -171 11-30	Sheet Rod
E(5)S-P(8)		42 - 218	Sheet
E(8)S-P(8)		37 - 207	Sheet
E(10)S-P(8)		85 – 331	Sheet
E(5)Am-P(10)Am		49 - 145	Sheet
E(8)Am- P(10)Am		93 – 218	Sheet
E(10)Am- P(10)Am		-	Platelet
E(5)S- P(10)Am		-	Sheet
E(8)S- P(10)Am		58 – 273	Sheet
E(10)S- P(10)Am		-	Sheet
E(5)Am-P(10)S		105 – 292	Sheet
E(8)Am- P(10)S		66 – 207	Sheet
E(10)Am- P(10)S		56 – 210 15 - 35	Sheet Rod
E(5)S- P(10)S		-	Sheet

E(8)S- P(10)S	350	-	Sheet
E(10)S- P(10)S		15-41	Rod
E(5)Am-P(10)Am 350		37- 266 21-61	Sheet Rod
E(10)Am-P(8) 350		40 – 340 29 - 47	Sheet Rod
E(10)Am- P(10)Am 350		Tube inner diameter 32 - 212 Tube wall Thickness 15 - 49	Tube
E(10)S- P(10)S 350		32-86	Rod

4.1.1.7 FTIR analysis

Figure 4.11 shows FTIR spectra of samples prepared initially using eggshell solution and diammonium hydrogen phosphate solution. Characteristic peaks of hydroxyapatite were identified in the range of $962 \sim 1022 \text{ cm}^{-1}$ and $559 \sim 601 \text{ cm}^{-1}$ [239] for both unwashed and washed samples. In addition, the unwashed samples showed some extra peaks corresponding to NH_4NO_3 which indicated its presence in the sample suspension as an impurity. The band positions and their respective assignments are given in Table 4.4.

Table 4.4: FT-IR band positions and their corresponding assignments of unwashed and washed samples.

Observed band positions (cm^{-1})		Corresponding assignments	References
Unwashed sample	Washed sample		
559	559	PO_4^{3-} bending (ν_4)	[240, 241]
601	601	PO_4^{3-} bending (ν_4)	[240, 241]
629	-----	Structural OH^-	[242, 243]
715	-----	NO_3^- in-plane deformation (ν_4)	[244]
827	-----	NO_3^- out-of-plane deformation (ν_2)	[244]
875	874	CO_3^{2-} bending (ν_2)	[95, 239, 242, 243, 245]

962, 1022, 1088	962, 1020	PO_4^{3-} bending (ν_1)	[95, 243]
1339-1539 1319, 1417	1358-1539 1452-1411	CO_3^{2-} bending (ν_3)	[95, 239, 242, 243, 245]
2874	-----	NH_4^+ asymmetric deformation (ν_4')	[244]
3061 – 3063	-----	NH_4^+ symmetric and asymmetric deformation ($\nu_2' + \nu_4'$)	[244]
3568	3566	Structural OH^-	[243, 246, 247]

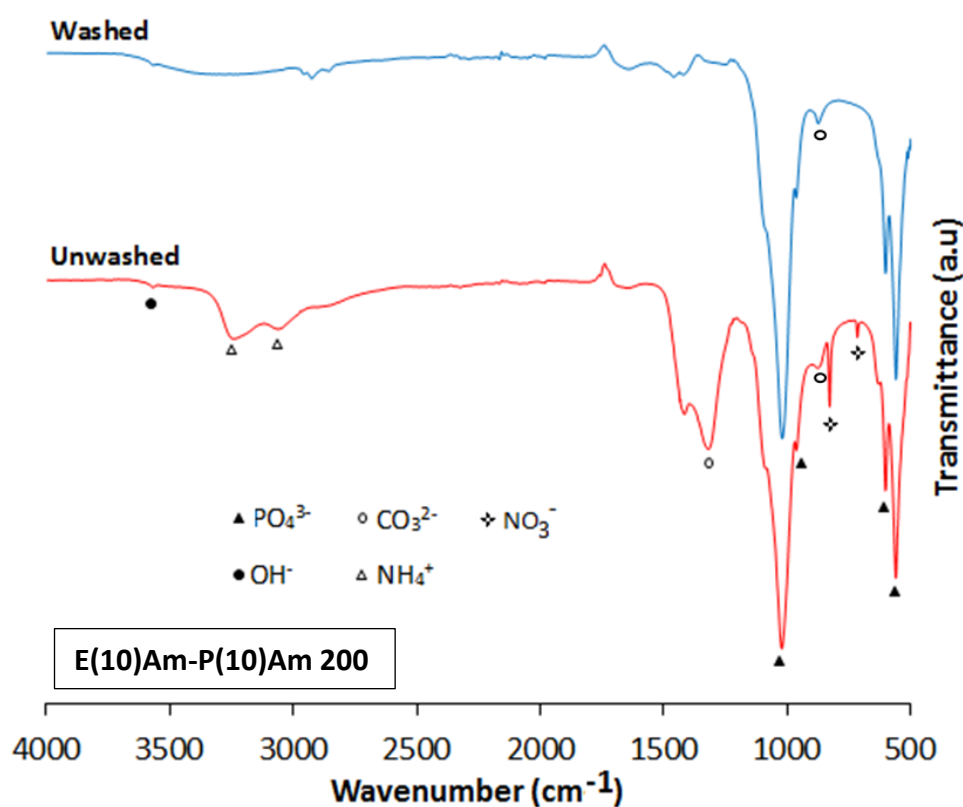


Figure 4.11: FTIR spectra of washed and unwashed samples of E(10)Am-P(10)Am 200 produced from eggshell solution and diammonium hydrogen phosphate solution via the hydrothermal synthesis process at 200 °C and pH 10.

To evaluate the effect of temperature and base solution on the structure of samples prepared, FTIR spectra of washed samples at two different temperatures (i.e. 200 and 350 °C)

using NH_4OH and NaOH as a base solution (maintaining pH at 10) are presented in Figure 4.12A and B respectively. Characteristic peaks of hydroxyapatite were identified in the range of $962 \sim 1022 \text{ cm}^{-1}$ and $559 \sim 601 \text{ cm}^{-1}$ [239] for both conditions and corresponding peaks to NH_4NO_3 were not observed.

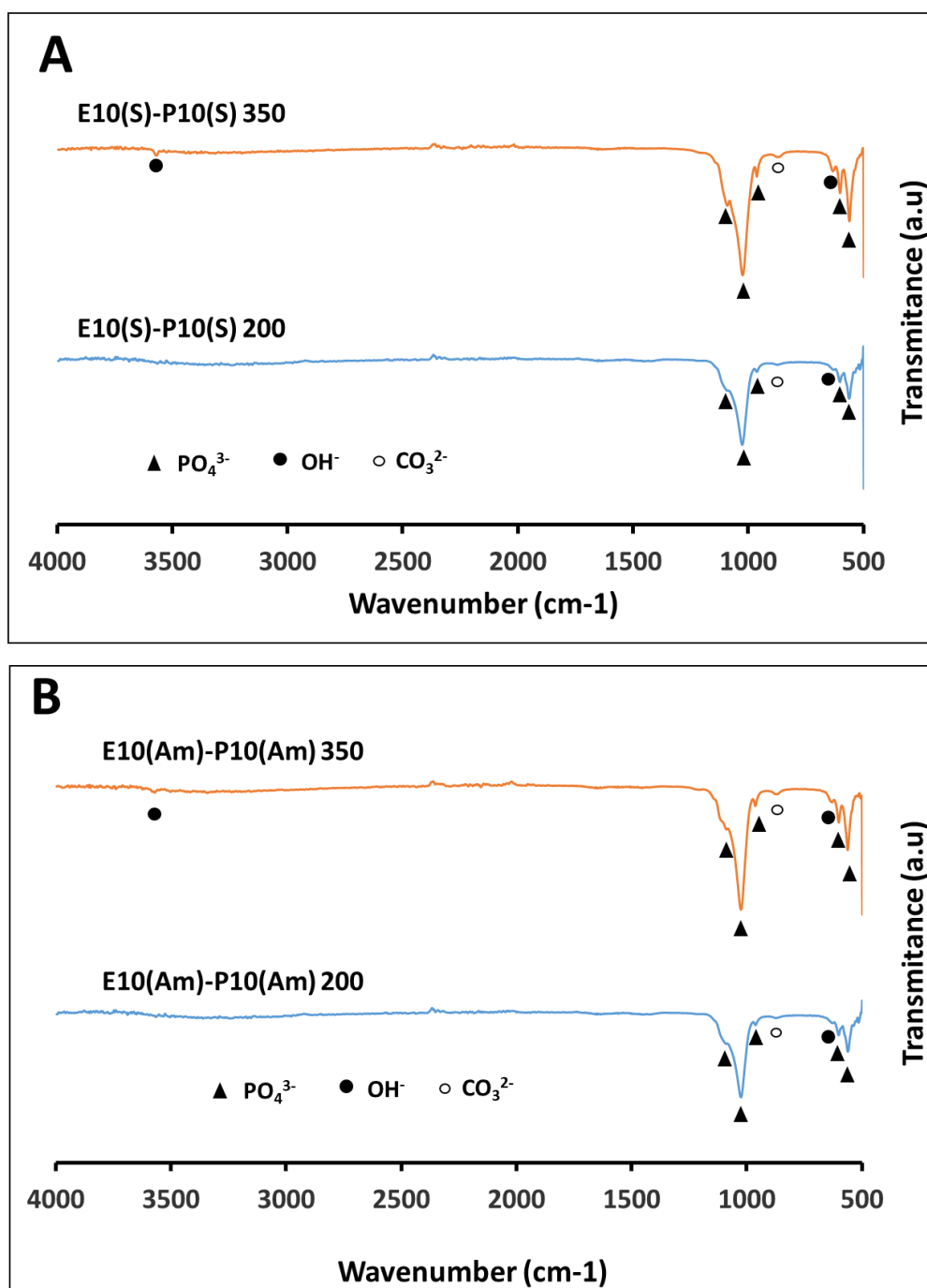


Figure 4.12: FTIR spectra of washed samples synthesised at 200 °C and 350 °C and at a constant pH of 10 maintaining using NH_4OH (A) and NaOH (B) via hydrothermal process.

4.1.2 Hydroxyapatite synthesis using calcium nitrate tetra-hydrate and struvite

Having confirmed hydroxyapatite (HA) formation was possible using eggshell solution (as the Ca source) in the first experimental section, struvite solution (used as the phosphate source) was introduced in the second set of experiments as an alternative potentially sustainable source for the hydrothermal synthesis process and calcium nitrate tetrahydrate solution was used as the Ca source here.

4.1.2.1 Investigating phase changes using XRD

Figure 4.13 represents the XRD pattern for the samples obtained from the initial trials of three sets of experiments at 200 °C and 350 °C, using struvite and calcium nitrate tetrahydrate solution of pH 6 and 11, respectively. Figure 4.13A and Figure 4.13B show the effect of two base solutions – NH_4OH and NaOH (which were used to adjust the pH of the precursors) on the products formed, where struvite was dissolved in HNO_3 . A mixture of HA and Mg-Whitlockite (Mg-WH) formed at 200 °C, whereas a single phase of Mg-WH was obtained at 350 °C when NH_4OH was used to adjust the pH (Figure 4.13A). However, semicrystalline Mg-WH formed at 200 °C when using NaOH as a base solution. Interestingly, solid particles obtained at 350 °C dissolved in the product solution within a few minutes (Figure 4.13B). Figure 4.13C shows the XRD pattern of the samples produced at 200 °C and 350 °C from struvite and calcium nitrate tetrahydrate solution, where acetic acid (CH_3COOH) was used to dissolve struvite (instead of HNO_3) to evaluate the effect of dissolution media on the products formed. A single-phase semicrystalline Mg-WH was obtained at 200 °C, while a mixture of Mg-WH and HA formed at 350 °C (Figure 4.13C). The crystalline phases identified were matched with HA (ICDD No. 00-009-0432) and Mg-WH (ICDD No. 00-042-0578).

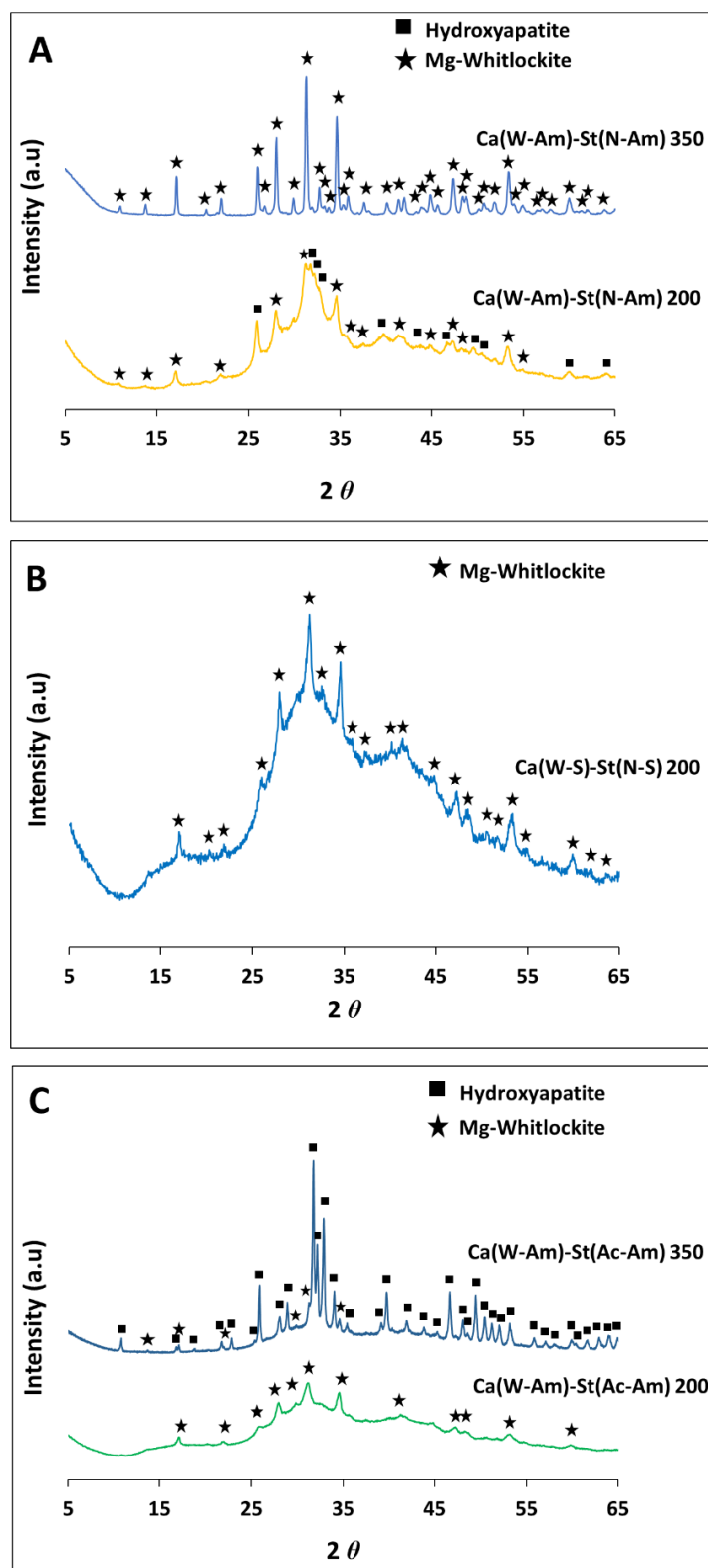


Figure 4.13: XRD pattern of samples synthesised at 200 °C and 350 °C via hydrothermal synthesis process at constant pH 11 of calcium nitrate tetrahydrate solution and pH 6 of struvite solution adjusted using **(A, C)** NH_4OH (Am) and **(B)** NaOH (S) individually where struvite was dissolved in **(A, B)**: HNO_3 (N) and **(C)** CH_3COOH (Ac). Calcium nitrate tetrahydrate was dissolved in DI Water (W).

4.1.2.2 Crystallographic parameters (Crystallite size, Crystallinity and percentage of phases)

Crystallographic parameters (i.e., crystal size and percentage of crystallinity) of the samples were produced at two different temperatures (i.e., 200 and 350 °C) using two different acids (i.e., HNO₃ and CH₃COOH to dissolve struvite) and base solutions (i.e., NH₄OH and NaOH to adjust the pH) are presented in Table 4.5. The crystallite size of HA and Mg-WH samples responsible for Bragg reflections of the (002) and (0210) planes respectively were calculated from XRD data using the Scherrer equation [94]. In all the experiments, the crystallite sizes of Mg-WH were found to be 62- 81 nm. The crystallite size of HA was found to be 17 nm for Ca(W-Am)-St(N-Am)200 where struvite was dissolved in HNO₃ and the experiment was conducted at 200 °C, whereas significantly higher crystallite size of HA (i.e., 80 nm) formed for Ca(W-Am)-St(Ac-Am)350 when struvite dissolved in CH₃COOH and experiment was operated at 350 °C. A similar values of crystallite size around 80-81 nm for most of the samples indicated that the crystallites were too large to produce peak broadening in the XRD patterns, so the crystallite size could no longer be calculated from the XRD data. However, repeat analysis of crystallographic parameters would be required for further confirmation.

The percentage crystallinity of the samples was found to be 7 – 11 % only at 200 °C whereas significantly higher crystallinity (i.e., 35 – 86 %) was observed for the samples synthesised at 350 °C.

The percentage of the phases (%) for the biphasic CaPs is also presented in Table 4.5. In Ca(W-Am)-St(N-Am) 200, the amount of HA and Mg-WH were found to be 51 % and 49 % respectively. Whereas, HA was found as a major phase (88 %) and Mg-WH as a minor phase (12 %) for the Ca(W-Am)-St(Ac-Am) 350.

Table 4.5: Crystallite size at (002) plane and (0210) for HA and Mg-WH, respectively and crystallinity and volume fraction (for the mixed phases) of samples prepared from calcium nitrate tetrahydrate solution and struvite solution via hydrothermal synthesis process at 200 and 350 °C.

Samples	Reaction temperature (°C)	Crystallite size (nm)		Crystallinity (%) (using background auto-correction)	Percentage of phases (%)	
		at the plane (002) for HA	at the plane (0210) for Mg-WH		HA	Mg-WH
Ca(W-Am)-St(N-Am)200	200	17	81	11	51	49
Ca(W-S)-St(N-S) 200		-	81	8	-	-
Ca(W-Am)-St(Ac-Am)200		-	81	7	-	-
Ca(W-Am)-St(N-Am)350	350	-	62	86	-	-
Ca(W-Am)-St(Ac-Am)350		80	81	35	88	12

4.1.2.3 Morphology characterisation using FEG-SEM-EDS and TEM

The surface morphology and the Ca/P ratio (atomic %) of the hydrothermally synthesised samples prepared from calcium nitrate tetrahydrate solution and struvite solution at 200 °C and 350 °C are presented in Figure 4.14. When HNO₃ was used to dissolve struvite and NH₄OH was used to adjust the pH of the precursor solutions, nanoparticles formed at 200 °C whilst larger particles (nano to submicron) were observed at higher temperature of 350 °C. Using NaOH as a base solution, nano-sized homogeneous Mg-WH particles were produced. On the other hand, preparing struvite solution using acetic acid and using NH₄OH as a base solution, Mg-WH particles with plate shaped morphology formed at 200 °C. Interestingly, tube morphology of HA along with rhombohedral-shaped Mg-WH particles was observed at higher temperature (i.e. 350 °C) in this system.

The ranges of Ca/P of the Mg-WH phase were found to be 1.23 – 1.45, while 1.58 and 1.77 were found for the HA phase. The ratios of Ca/P, Ca+Mg/P and Mg/Ca of the samples prepared are presented in Table 4.6. The range of the ratios for the samples Ca(W-Am)-St(N-Am)200 and Ca(W-Am)-St(Ac-Am)350 revealed the presence of Mg-WH along with HA.

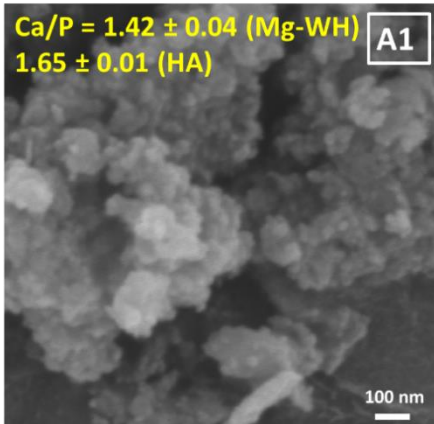
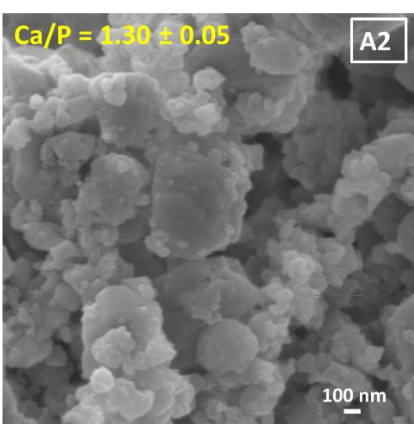
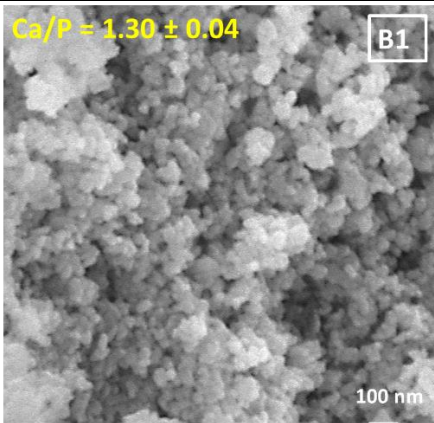
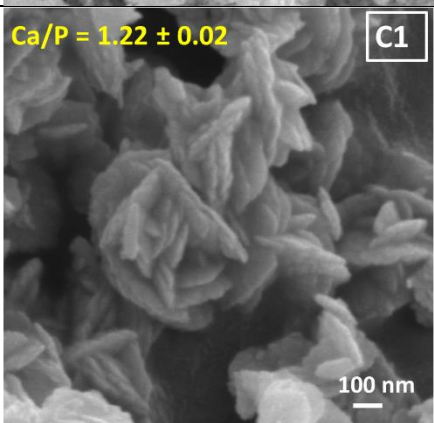
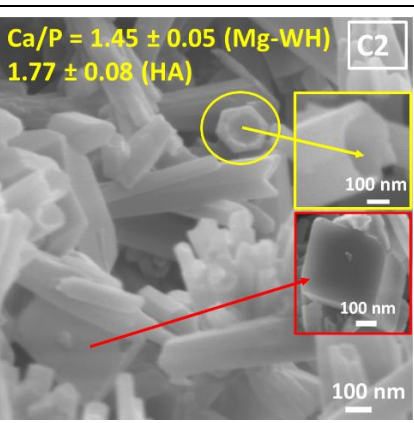
Samples name	Acid to dissolve Struvite	Base solution to adjust pH	Temperature	
			200 °C	350 °C
Ca(W-Am)-St(N-Am)	HNO ₃	NH ₄ OH	Ca/P = 1.42 ± 0.04 (Mg-WH) 1.65 ± 0.01 (HA) A1 	Ca/P = 1.30 ± 0.05 A2 
Ca(W-S)-St(N-S)		NaOH	Ca/P = 1.30 ± 0.04 B1 	No product formed
Ca(W-Am)-St(Ac-Am)	CH ₃ COOH	NH ₄ OH	Ca/P = 1.22 ± 0.02 C1 	Ca/P = 1.45 ± 0.05 (Mg-WH) 1.77 ± 0.08 (HA) C2 

Figure 4.14: Surface morphology and Ca/P ratio (atomic) of samples synthesised using Calcium nitrate tetrahydrate solution (pH=11) and Struvite solution (pH=6) at 200 °C and 350 °C where HNO₃ (A1, A2 and B1) and CH₃COOH (C1 and C2) were used to dissolve struvite and NH₄OH (A1, A2, C1 and C2) and NaOH (B1) were used to adjust the pH of the precursor solutions. Yellow colour highlights the tube morphology and the red colour highlights a rhombohedral-shaped particle (C2).

Table 4.6: The ratios of Ca/P, Ca+Mg/P and Mg/Ca of the samples prepared from calcium nitrate tetrahydrate solution and struvite solution via hydrothermal synthesis process at 200 and 350 °C.

Samples	Ca/P	Ca+Mg/P	Mg/Ca
Ca(W-Am)-St(N-Am) 200	1.42 – 1.65	1.47 – 1.83	0.03 - 0.07
Ca(W-S)-St(N-S) 200	1.3	1.4	0.14
Ca(W-Am)-St(Ac-Am) 200	1.22	1.39	0.15
Ca(W-Am)-St(N-Am) 350	1.3	1.4	0.1
Ca(W-Am)-St(Ac-Am) 350	1.45 - 1.77	1.5 - 1.74	0.02 – 0.09

The morphologies of the samples obtained, characterised by TEM are shown in Figure 4.15. Nano-sized irregular-shaped particles along with some plate-shaped particles were observed at 200 °C for Ca(W-Am)-St(N-Am) 200 (Figure 4.15A) while larger rhombohedral-shaped particles were observed at higher temperature (i.e. 350 °C, Figure 4.15B). TEM images for the sample Ca(W-S)-St(N-S) 200 were found to be agglomerated, where the rhombohedral shaped particles along with nano-sized irregular particles were observed (Figure 4.15C). Figure 4.15D and Figure 4.15E show the morphologies of samples (obtained using acetic acid to dissolve struvite and NH₄OH to adjust the pH) at different tilting positions (± 30 degree angle). The particles highlighted in yellow circles for 2 different angles clearly indicated platelet-like morphology for the samples produced at 200 °C (Ca(W-Am)-St(Ac-Am) 200) and mixed morphology of tube and rhombohedral for the samples produced at 350 °C (Ca(W-Am)-St(Ac-Am) 350).

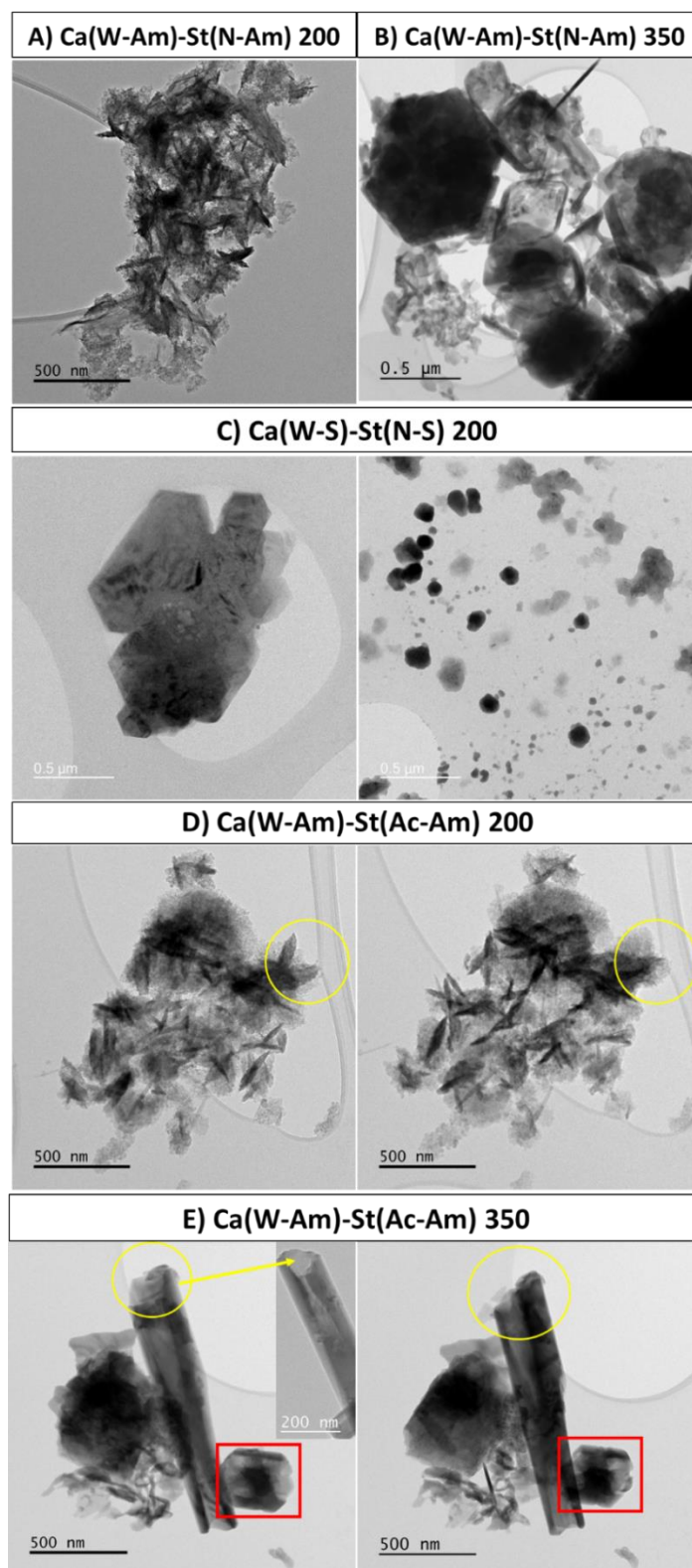


Figure 4.15: TEM images of sample Ca(W-Am)-St(N-Am)200 prepared (using HNO_3 to dissolve struvite, NH_4OH to adjust the pH) at A) 200 $^{\circ}\text{C}$, B) 350 $^{\circ}\text{C}$ and C) images for Ca(W-Am)-St(N-Am)350 (using HNO_3 to dissolve struvite, NaOH to adjust the pH) at 200 $^{\circ}\text{C}$. TEM images (at tilting positions; ± 30 degree angle) of samples prepared (using

HNO₃ to dissolve struvite, NH₄OH to adjust the pH) at D) 200 °C and E) 350 °C from calcium nitrate tetrahydrate solution (pH 11) and struvite solution (pH 6). (D) Yellow circles highlight platelet-shaped particles. (E) Yellow colour highlights the tube morphology and red colour highlights a rhombohedral-shaped particle.

4.1.2.4 Particle Size analysis from FEG-SEM images

The particle size of the Ca(W-Am)-St(Ac-Am) 350 was measured from SEM images via ImageJ software. 20 particles were considered for this measurement. Particle sizes of that sample were found to be 50-160 nm in diameter with 20-40 nm thickness of the tube and 23 – 720 nm of rhombohedral.

The particles of the rest of the samples prepared were found to be agglomerated as seen from SEM images (Figure 4.14) and therefore, their particle sizes could not be accurately measured.

4.1.2.5 Functional group analysis by FTIR

The FTIR spectra of samples prepared in three sets of experiments are shown in Figure 4.16. The characteristic peaks related to different functional groups such as PO₄³⁻, structural OH⁻, HPO₄²⁻ and CO₃²⁻ for all the samples prepared are presented in Table 4.7. The four characteristic bands related to PO₄³⁻ were identified in the range of 960 -1087 cm⁻¹ and 547-603 cm⁻¹ [239] for all the samples prepared except the sample Ca(W-S)-St(N-S) 200 (obtained by using NaOH as a base solution) which showed only two characteristic bands of PO₄³⁻ at 551 and 1022 cm⁻¹. A notable structural OH⁻ band was observed at 629 cm⁻¹ and 3570 cm⁻¹ for the sample Ca(W-Am)-St(Ac-Am) 350. The characteristic bands of HPO₄²⁻ at around 870 cm⁻¹ were observed for all the samples produced, while the band at around 920 cm⁻¹ (related to HPO₄²⁻) was identified only for the samples (Ca(W-Am)-St(N-Am) 350 and Ca(W-Am)-St(Ac-Am) 350) obtained at higher temperature (350 °C). All the samples also showed a band related to CO₃²⁻ bending at around 1640 cm⁻¹. The broad band in the range of 3000 – 3600 cm⁻¹ corresponding to absorbed water was identified for all the samples except Ca(W-Am)-St(Ac-Am) 350. In addition, a peak at 825 cm⁻¹ corresponding to NO₃⁻ was found for the samples produced at higher temperature (i.e., 350 °C).

Table 4.7: FT-IR band positions and their corresponding assignments of samples produced at 200 °C and 350 °C from calcium nitrate tetrahydrate and struvite solutions.

Observed band positions (cm ⁻¹)					Corresponding assignments	References
Ca(W-Am)- St(N-Am) 200	Ca(W-Am)- St(N-Am) 350	Ca(W-S)- St(N-S) 200	Ca(W-Am)- St(Ac-Am) 200	Ca(W-Am)- St(Ac-Am) 350		
557	553	551	547	559	PO ₄ ³⁻ bending (ν_4)	[240, 241, 248, 249]
602	603	-	603	600	PO ₄ ³⁻ bending (ν_4)	[240, 241, 248-250]
-	-	-	-	629	structural OH ⁻	[249, 251]
-	825	-	-	825	NO ₃ ⁻ out-of-plane deformation (ν_2)	[244, 249]
877	889	870	881	874	HPO ₄ ⁻ group, / B-site CO ₃ ²⁻ bending (ν_3)/ (ν_2)	[95, 239, 242, 243, 245, 248, 249, 252]
-	922	-	-	925	HPO ₄ ⁻ group	[252]
962	960	-	-	962	PO ₄ ³⁻ bending (ν_1)	[95, 243, 248-250]
1024	991, 1011	1022	1011	1020	PO ₄ ³⁻ bending (ν_3)	[95, 243, 250]
-	1059	-	1070	1087	PO ₄ ³⁻ bending (ν_3)	[95, 243, 250]
-	1340	-	-	1338	CO ₃ ²⁻ bending (ν_3)	[95, 239, 242, 243, 245]
	1425	-	-	1417	B-site CO ₃ ²⁻ bending (ν_3)	[95, 239, 242, 243, 245]
-	-	-	-	1552-1554	A-type CO ₃ ²⁻ bending	
1638	1641	1649	1641	1641	absorbed water	[248, 249]
3100 - 3500	3100 - 3500	3000 - 3600	3100 - 3500	-	Absorbed water	[249]
-	-		-	3570	Structural OH ⁻	[249]

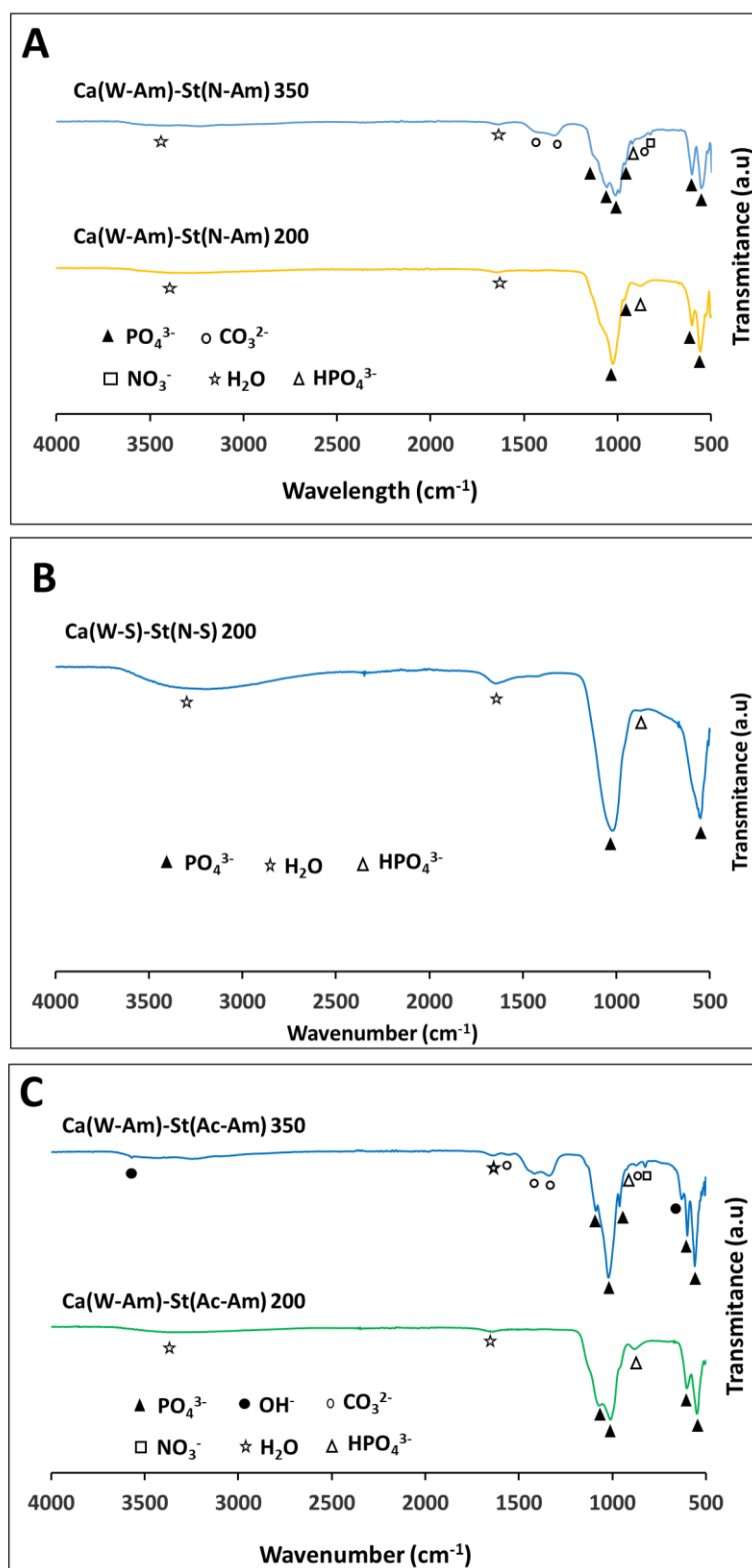


Figure 4.16: FTIR spectra of samples synthesised at 200 °C and 350 °C and at constant pH 11 and 6 for Calcium nitrate tetrahydrate solution and Struvite solution, respectively where acid and base solutions were (A) HNO_3 and NH_4OH , (B) HNO_3 and NaOH , (C) CH_3COOH and NH_4OH , respectively.

4.1.3 Hydroxyapatite synthesis using eggshell and struvite

After having shown that HA formation was possible using eggshell solution (as the Ca source) and di-ammonium hydrogen phosphate (as the P sources) in the first experiments, and mixed-phase of Mg-WH and HA or single phase of Mg-WH obtained using calcium nitrate tetrahydrate (as Ca source) and struvite (as the P source) in the second experiments, both sustainable sources of Ca (i.e eggshells) and P (in struvite form) were introduced into the hydrothermal synthesis process and the output is presented here in the third set of experiments.

4.1.3.1 Investigating phase changes using XRD

The XRD pattern of the samples produced from the two sustainable precursor solutions (eggshell solution and struvite solution) at 200 °C and 350 °C are presented in Figure 4.17A-C. When nitric acid was used to dissolve both precursors, the pH of all the precursor solutions (pH=11 for eggshell solutions and pH=6 for struvite solutions) were maintained using two different base solutions (i.e. NH_4OH and NaOH), and their effects on the products formed are shown in Figure 4.17A and B. A well-pronounced background was observed for all the obtained XRD patterns. This observation revealed that the developed samples [i.e., E(N-Am)-St(N-Am) 350 and E(N-S)-St(N-S) 200] mostly consisted of an amorphous phase along with a minor amount of crystalline phase except E(N-Am)-St(N-Am) 200, which formed only amorphous calcium phosphate (ACP) at 200 °C. A small amount of mixed-phase of HA and Mg-WH were observed for E(N-Am)-St(N-Am) 350 at 350 °C (Figure 4.17A) and a single phase of Mg-WH formed in a minimal amount at 200 °C for E(N-S)-St(N-S) 200 (Figure 4.17B).

Figure 4.17C shows the XRD pattern of the samples produced at 200 and 350 °C, where acetic acid was used to dissolve eggshells and struvite (instead of HNO_3) to evaluate the effect of acid on the samples formed. The pH of both solutions was adjusted using NH_4OH . A single-phase of HA was obtained at 200 °C, whereas a multiphase of Mg-WH and HA formed at 350 °C (Figure 4.17C). The crystalline phases identified were matched with HA (ICDD No. 00-009-0432) and Mg-WH (ICDD No. 00-042-0578).

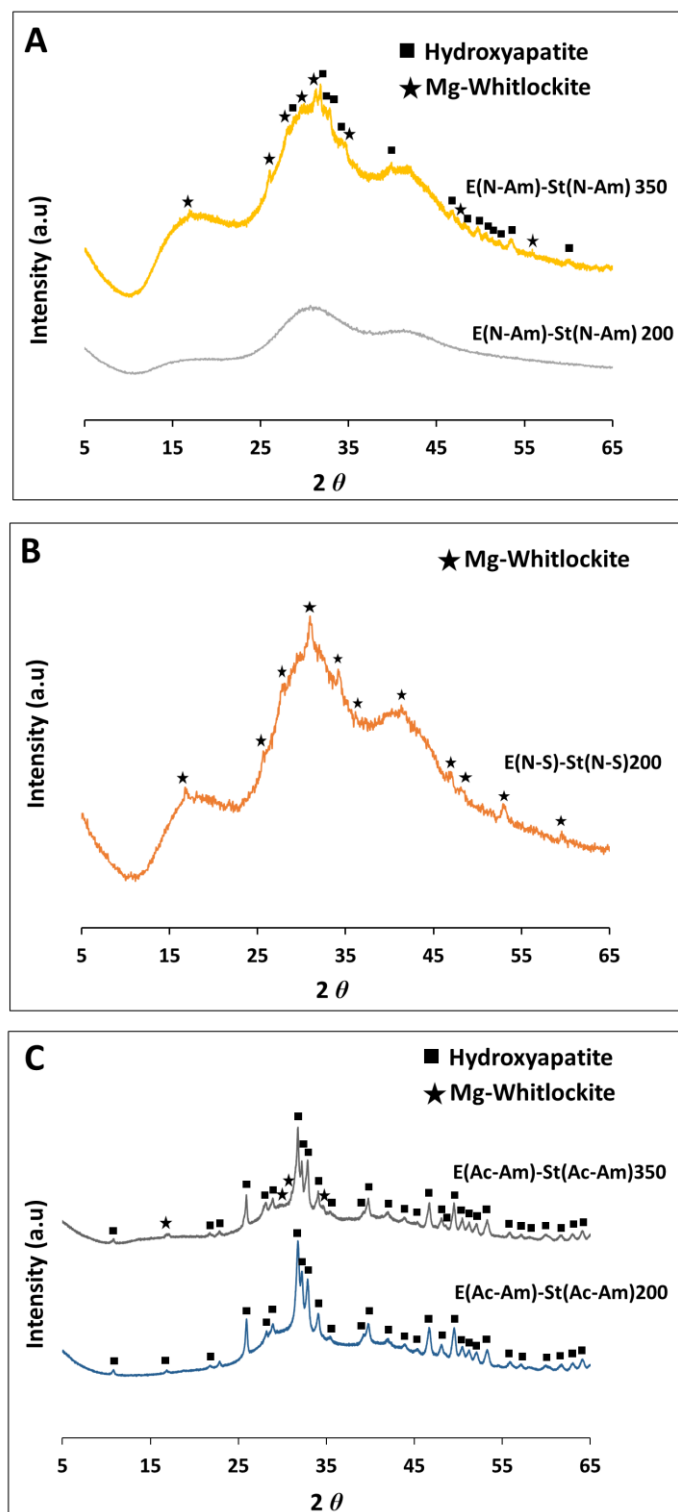


Figure 4.17: XRD pattern of samples synthesised at 200 °C and 350 °C via hydrothermal synthesis process at constant pH 11 of Eggshell solution and pH 6 of Struvite solution adjusted using (A, C) NH_4OH (Am) and (B) $NaOH$ (S) individually where eggshells and struvite were dissolved in (A, B) HNO_3 (N) and (C) CH_3COOH (Ac).

4.1.3.2 Crystallographic parameters (crystallite size, crystallinity and volume fraction)

The crystallite size and crystallinity of the samples prepared in the three sets of experiments and the volume fraction (%) of the phases of the samples containing multiphase are shown in Table 4.8. The crystallite size of HA and Mg-WH samples responsible for Bragg reflections of the (002) and (0210) planes, respectively were calculated from XRD data using the Scherrer equation [94]. Similar crystallite sizes of 81 and 82 nm were observed for HA and Mg-WH, respectively, which indicated that the crystallites were too large to produce peak broadening in the XRD patterns as mentioned in the previous section, so the crystallite size could no longer be calculated from the XRD data. However, repeat analysis of crystallographic parameters would also be required for these samples for further confirmation.

However, only a very low percentage of crystallinity (i.e., 2 - 6%) was found for the samples formed when, precursors were dissolved in HNO_3 and NH_4OH (Figure 4.17A) and NaOH (Figure 4.17B) used as a base solution, whereas significantly higher crystallinity (i.e., 19 – 20%) was observed at both temperatures (i.e., 200 °C and 350 °C) for the samples produced using CH_3COOH to prepare precursor solutions.

Samples containing multiphase were observed to be formed at higher temperature (i.e., 350 °C). 49 % of Mg-WH and 51 % of HA was found for the samples produced using HNO_3 to dissolve precursors. On the other hand, HA formed as a major phase (81 %) with a minor phase of Mg-WH (19 %) when CH_3COOH was used to dissolve the precursors.

Table 4.8: Crystallite size at (002) plane and (0210) for HA and Mg-WH, respectively and crystallinity and volume fraction (for the mixed phases) of samples prepared from eggshell solution and struvite solution via hydrothermal synthesis process at 200 and 350 °C.

Samples	Reaction temperature (°C)	Crystallite size (nm)		Crystallinity (%) (using background auto-correction)	Volume fraction (%)	
		at plane (002) for Hydroxyapatite (HA)	at plane (0210) for Mg-Whitlockite (Mg-WH)		HA	Mg-WH
E(N-Am)-St(N-Am) 200	200	-	-	-	-	-
E(N-S)-St(N-S) 200		-	82	6	-	-
E(Ac-Am)-St(Ac-Am) 200		81	-	19	-	-
E(N-Am)-St(N-Am) 350	350	81	82	2	51	49
E(Ac-Am)-St(Ac-Am) 350		81	82	20	81	19

4.1.3.3 Morphology characterisation using FEG-SEM-EDS and TEM

The surface morphology and the Ca/P ratio (atomic %) of the hydrothermally synthesised samples prepared from eggshell solution and struvite solution at 200 °C and 350 °C are presented in Figure 4.18. When HNO₃ was used to dissolve precursors and NH₄OH to adjust the pH of the precursors solutions, spherical shaped particles were observed at 200 °C (Figure 4.18A1), whereas only a few crystals of mixed morphologies (i.e., Ellipsoidal, tube, and rhombohedral) of HA and Mg-WH were found at higher temperature (i.e., 350 °C) (Figure 4.18A2). Irregular-shaped nanoparticles including some rhombohedral crystals of Mg-WH were observed at 200 °C when NaOH was used as a base solution (Figure 4.18B1). Mixed morphologies of HA and/or Mg-WH were also observed for the samples obtained at both

temperatures (i.e 200 and 350 °C) using CH_3COOH and NH_4OH to dissolve precursors and adjust the pH, respectively. Larger particles (nano to submicron) of Ellipsoidal and tube (yellow circled) shaped of HA were observed at 200 °C (Figure 4.18C1), whereas rod and tube-shaped (yellow circled) HA and rhombohedral (red circled and single crystal image in the inset) shaped Mg-WH particles were found to form at 350 °C (Figure 4.18C2). The ranges of Ca/P of the Mg-WH phase were found to be 1.23 – 1.45, while 1.58 and 1.77 were found for the HA phase.

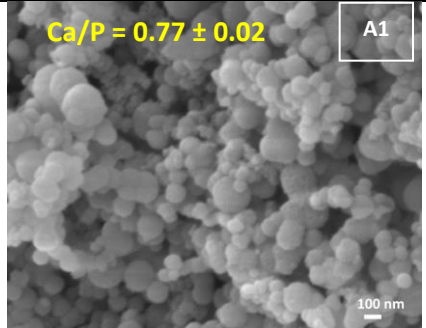
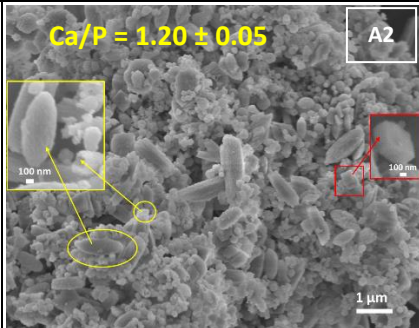
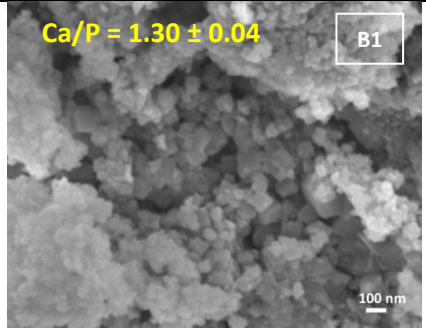
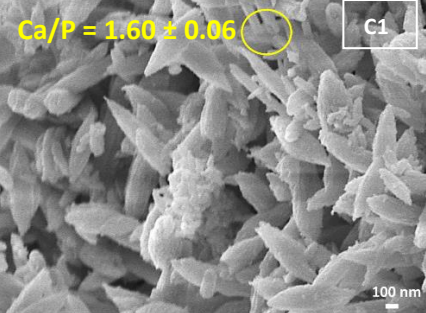
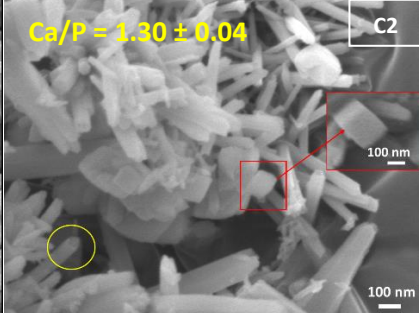
Samples name	Acids to dissolve the eggshell solution	Acids to dissolve Struvite	Base solution to adjust pH	Temperature	
				200 °C	350 °C
E(N-Am)-St(N-Am)	HNO_3	HNO_3	NH_4OH		
E(N-S)-St(N-S)	HNO_3	HNO_3	NaOH		No product obtained
E(Ac-Am)-St(Ac-Am)	CH_3COOH	CH_3COOH	NH_4OH		

Figure 4.18: Surface morphology and Ca/P ratio (atomic) of samples synthesised using Eggshell solution (pH=11) and Struvite solution (pH=6) at 200 °C and 350 °C, where HNO_3 (A1, A2 and B1) and CH_3COOH (C1 and C2) were used to dissolve eggshells and struvite and NH_4OH (A1, A2, C1 and C2) and NaOH (B1) were used to adjust the pH of the precursor solutions. The elliptical and rod-shaped HA are highlighted in yellow colour and the rhombohedral-shaped Mg-WH is highlighted in red colour.

The morphologies of the samples obtained, characterised by TEM are shown in Figure 4.19. From the TEM images, elliptical, tube, and rhombohedral particles in the amorphous system were clearly observed for sample E(N-Am)-St(N-Am) 350 (Figure 4.19). Nano-sized irregular-shaped particles for the sample E(N-S)-St(N-S) 200 were also seen in the TEM image (Figure 4.19B).

Figure 4.19C and D show the morphology of samples obtained using acetic acid to dissolve precursors and NH_4OH to adjust the pH. Elliptical-shaped particles were observed at lower temperature (Figure 4.19C), whilst a mixed morphology of rhombohedral and rods/tubes were identified for the samples produced at higher temperature (Figure 4.19D).

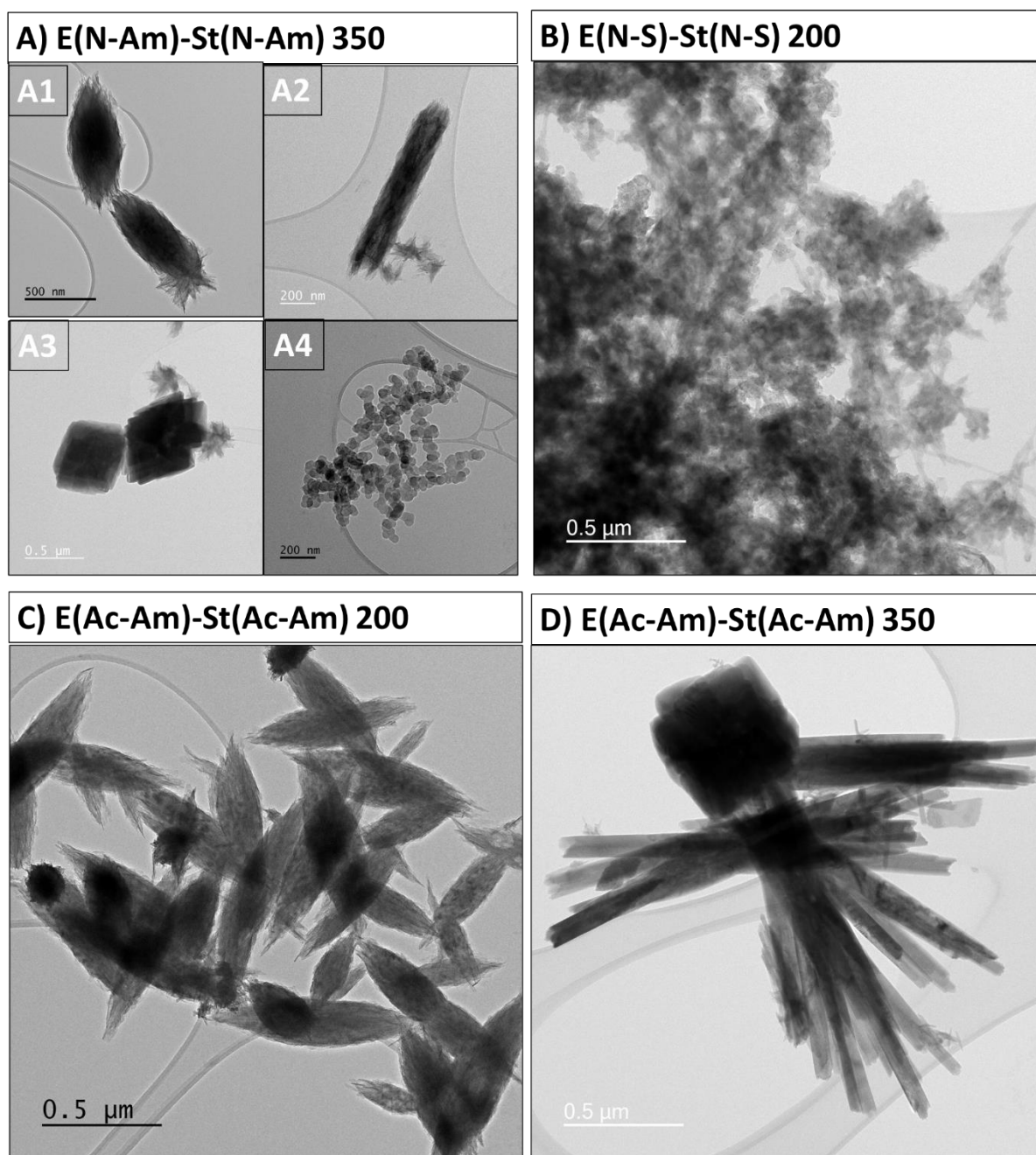


Figure 4.19: TEM images of sample A) E(N-Am)-St(N-Am)350 prepared (using HNO_3 to dissolve precursors, NH_4OH to adjust the pH) at 350 °C, B) E(N-S)-St(N-S)200 prepared (using HNO_3 to dissolve Eggshell and Struvite, NaOH to adjust the pH) at 200 °C and C) TEM images (at tilting positions; ± 30 degree angle) of E(Ac-Am)-St(Ac-Am) 200 prepared (using CH_3COOH to dissolve precursors, NH_4OH to adjust the pH) at 200 °C.

4.1.3.4 Particle Size analysis from FEG-SEM images

The particle size range of the samples measured from FEG-SEM images via ImageJ software is presented in Table 4.9. It can be observed from the table that most of the samples were nano to submicron range. The particle size of ACP obtained at 200 °C was in the range of 28 – 196 nm. Elliptical shaped particles of E(Ac-Am)-St(Ac-Am) 200 were found to be 96 – 273 nm. At higher temperature (i.e, 350 °C), samples obtained using HNO₃ were found to be contained Ellipsoidal particles of 273 – 522 nm in width; 238 – 278 nm of rhombohedral, and tube with an inner diameter of 99 – 290 nm and thickness of 28 – 71 nm, whereas using CH₃COOH to dissolve precursors produced 37- 266 nm tube and 96 – 214 nm rhombohedral. Particle sizes of E(N-S)-St(N-S) 200 samples could not be measured due to agglomeration.

Table 4.9: Particle size range of samples prepared from Eggshells solution and Struvite solution via hydrothermal synthesis process at 200 and 350 °C.

Samples name	Reaction temperature (°C)	Particle size range (nm)	Morphology
E(N-Am)-St(N-Am) 200	200	28 - 196	Spherical
E(N-S)-St(N-S) 200		-	Irregular-shaped and rhombohedral
E(Ac-Am)-St(Ac-Am) 200		96 - 273	Ellipsoidal
E(N-Am)-St(N-Am) 350	350	Width 273 – 522 238 – 278 inner diameter 99 – 290 thickness 28 - 71	Ellipsoidal Rhombohedral Tube
E(Ac-Am)-St(Ac-Am) 350		37- 266 96 – 214	Rod, Tube Rhombohedral

4.1.3.5 Functional group analysis by FTIR

Figure 4.20A-C represent the FTIR spectra of samples prepared in three sets of experiments.

The characteristics peak related to different functional groups such as PO_4^{3-} , structural OH^- , HPO_4^{2-} and CO_3^{2-} for all the samples prepared are presented in Table 4.10.

All the samples prepared showed four characteristic bands of PO_4^{3-} in the range of 550-604 cm^{-1} and 960 -1092 cm^{-1} [239]. However, the sample (E(N-Am)-St(N-Am)200) only showed two characteristic bands of PO_4^{3-} at 561 cm^{-1} and 1022 cm^{-1} . A notable band of structural OH^- was observed at 3572 cm^{-1} for the sample E(Ac-Am)-St(Ac-Am) 350. Samples prepared using acetic acid as a dissolution media showed bands of HPO_4^{2-} / CO_3^{2-} bending (ν_3) at around 870 cm^{-1} . A broad band of absorbed water at 3000 – 3600 cm^{-1} was identified for the samples E(N-Am)-St(N-Am)200 and E(N-S)-St(N-S) 200.

Table 4.10: FT-IR band positions and their corresponding assignments of samples produced at 200 °C and 350 °C from eggshell and struvite solutions.

Observed band positions (cm^{-1})					Corresponding assignments	References
E(N-Am)-St(N-Am) 200	E(N-Am)-St(N-Am) 350	E(N-S)-St(N-S) 200	E(Ac-Am)-St(Ac-Am) 200	E(Ac-Am)-St(Ac-Am) 350		
561	557	550	561	561	PO_4^{3-} bending (ν_4)	[240, 241, 248, 249]
-	600	604	601	602	PO_4^{3-} bending (ν_4)	[240, 241, 248-250]
-	-	-	-	619	structural OH^-	[249, 251]
-	-	-	874	877	HPO_4^{2-} group, CO_3^{2-} bending (ν_3)	[95, 239, 242, 243, 245, 248, 249, 252]
-	-	920	-	-	HPO_4^{2-} group	[252]

-	-	962	960	960	PO_4^{3-} bending (ν_1)	[95, 243, 248-250]
1022	1020	1014	1022	1022	PO_4^{3-} bending (ν_3)	[95, 243, 250]
-	-	1070	-	1092	PO_4^{3-} bending (ν_3)	[95, 243, 250]
-	-	1423	1420, 1454	1423, 1452	CO_3^{2-} bending (ν_3)	[95, 239, 242, 243, 245]
-	-	-	-	1562	CO_3^{2-} bending (ν_3)	[248, 249]
1651, 3000 - 3600	-	1649, 3000 - 3600	-	-	Absorbed water	[249]
-	-	-	-	3572	Structural OH^-	[249]

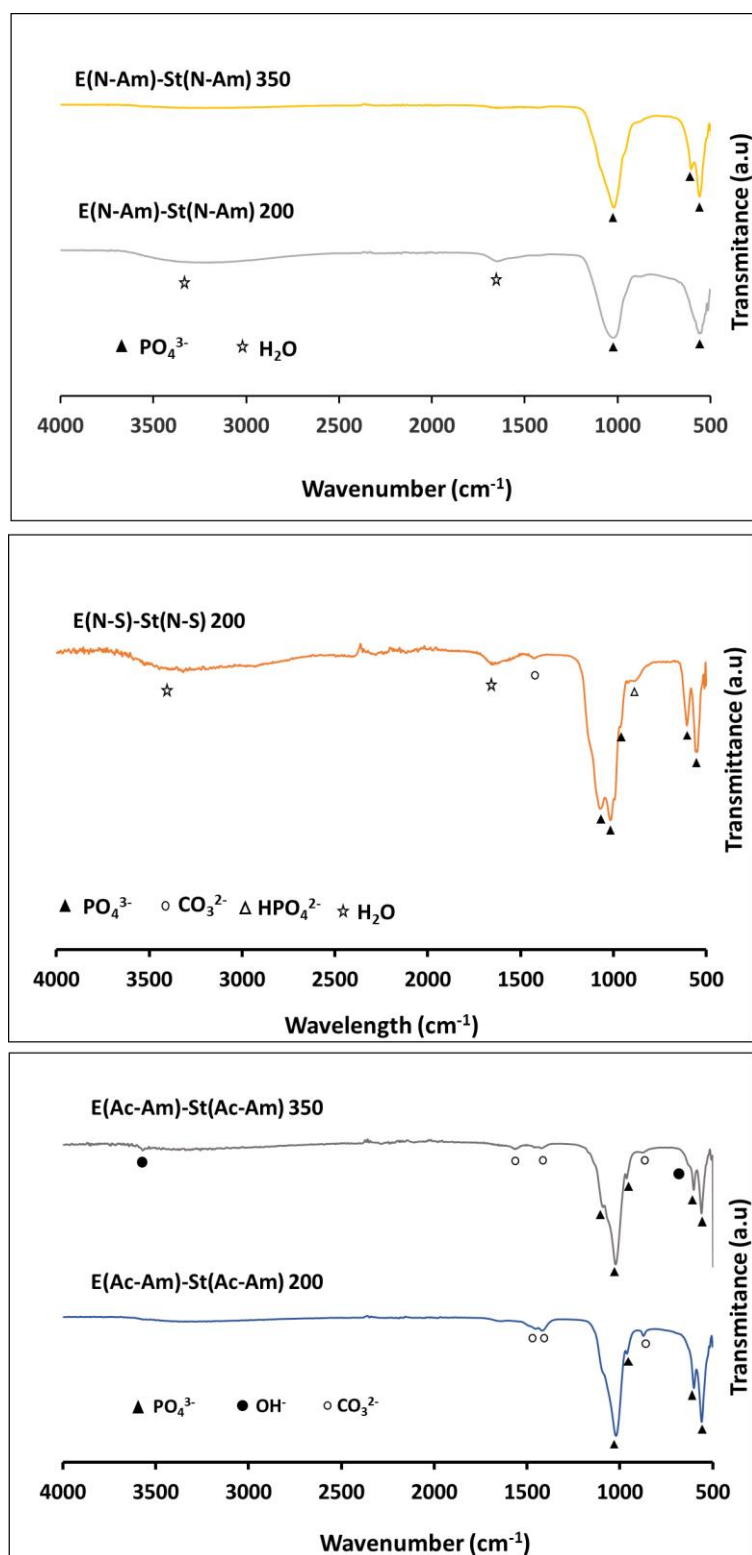


Figure 4.20: FTIR spectra of samples synthesised at 200 °C and 350 °C and at constant pH 11 and 6 for eggshell solution and struvite solution, respectively where acid and base solutions were (A) HNO_3 and NH_4OH , (B) HNO_3 and NaOH , (C) CH_3COOH and NH_4OH , respectively.

4.2 Discussion

Waste materials, from varying sectors such as farming or food processing, create adverse environmental effects such as soil, water or air pollution [253]. However, if some of these waste materials could be re-used this would not only protect the environment but could also provide a valuable raw resource for new materials development. For example, waste materials such as mammalian and fish bones, eggshells, seashells, plants and algae could all potentially be re-used. For instance, eggshell-derived hydroxyapatite (HA) has been shown to be more biocompatible with human bone due to the presence of some trace elements like Na, Mg, P, Sr and Zn in eggshells [94, 107]. Also compared to HA synthetically produced, eggshell-derived HA showed superior 'sinterability' [1] and osteoconduction [108-110] which would also be beneficial to the overall physiological performance after implantation.

Likewise, direct disposal of P-rich wastes from farms, municipal wastes and industrial processes (i.e. production of chemicals, food and beverages, pharmaceuticals, tannery, textiles, semiconductor, potato processing and molasses based food processing) [98] can cause eutrophication, subsequently resulting in contamination of fresh water streams [11]. Therefore, recovering phosphorus from wastewater streams would be beneficial for waste management strategies which could potentially be done through the precipitation of struvite (magnesium ammonium phosphate; $\text{MAP}/\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) [11, 12, 98, 114]. Kataki *et al.* [98] reported that some commercially available struvite recovery technologies were becoming available (i.e. Pearl[®] and/or ASSTRIP[®] (USA), Phospaq[™] (The Netherlands), Phosnix (Japan), Seaborne (Germany), AirPrex[™] (Germany, Netherlands), Multiform (America)) with 80-90% P recovery.

As such, struvite would be a potential P source for sustainable HA preparation addressing the circular economy. However, to the best of the authors knowledge, no research has been reported on CaP preparation from struvite as a P source.

In this section two different waste-derived materials (i.e. eggshells and struvite) were selected as Ca and P sources to explore synthesis of CaPs via a continuous hydrothermal synthesis process, with a view towards sustainable manufacture of biomaterials. The effect of varying pH, base solutions (used to maintain pH) and temperature on the morphology of the materials produced was also evaluated. A study by Lester *et al.* exploring synthesis of nano HA at

varying pH levels, base solutions, dissolution media and temperature via hydrothermal synthesis reported that the morphology of nano HA could be altered by varying these parameters [228]. In the first part of this chapter, one precursor material derived from waste resources (i.e. eggshells as Ca source) and another chemically derived (synthetic) precursor of P source (i.e. diammonium hydrogen phosphate) were used to prepare HA via hydrothermal synthesis and their chemical and morphological properties were evaluated via XRD, FTIR, FEG-SEM, EDX, TEM and TGA.

4.2.1 Samples prepared using Eggshell solution and Diammonium hydrogen phosphate solution

HA was successfully prepared from eggshell solution and diammonium hydrogen phosphate solution via the continuous flow hydrothermal synthesis process, which was confirmed via XRD (Figure 4.2) and FTIR (Figure 4.11 and Table 4.4). The unwashed HA contained residuals of NH_4NO_3 salt which was established via XRD and FTIR analysis [244]. This was due to the presence of NH_4NO_3 in the HA suspension as a by-product of the reaction between eggshell solution and diammonium hydrogen phosphate solution [248]. This suggested that a further wash step would be required for the samples prepared.

From FTIR analysis (Figure 4.11) of unwashed and washed samples, the peaks for stretching vibration modes of $\nu_1\text{-PO}_4^{3-}$ (962 cm^{-1}), $\nu_3\text{-PO}_4^{3-}$ (1020 cm^{-1}), $\nu_4\text{-PO}_4^{3-}$ (559 and 601 cm^{-1}) and stretching of OH^- group (3568 and 3566 cm^{-1}) were identified which represented characteristics peaks of HA [246, 247, 254]. Hamidi *et al.* [254] investigated a comparative study on synthesis and characterisation of eggshell-derived hydroxyapatite via a mechanochemical method. They reported that the peak of the OH^- group, known as the characteristic peak of HA was detected at 3569 cm^{-1} . However, in this present study, characteristic peaks of the substitutional group CO_3^{2-} were detected at 1339 , 1500 cm^{-1} and at 875 cm^{-1} for both samples. The stretching vibrations of the CO_3^{2-} bands at these points suggested that the phosphate group of the apatite was partially replaced by the carbonate group occurring B-type substitution, which plays a vital role in bioactivity of HA [248, 255]. The incorporation of the CO_3^{2-} into the HA structure may have been due to the existence of trace amounts of CaCO_3 found in the samples prepared [246, 254]. Hamidi *et al.* [254] also

reported the existence of absorption spectra of CO_3^{2-} in between $1460\text{--}1420\text{ cm}^{-1}$ (which has been reported as one of characteristic peaks of biological apatite) and attributed this to the presence of trace CaCO_3 from eggshell powder. They also reported non-stoichiometric HA for the samples they prepared and suggested that these samples were similar to the structure of biological apatite due to the presence of CO_3^{2-} groups [254]. This finding was also supported by another study by Ofudje *et al.* [255], who prepared HA from pig bone waste via thermal treatment between 600 and $1000\text{ }^\circ\text{C}$ and found the stretching vibrations of CO_3^{2-} bands at 870 and 1466 cm^{-1} for their samples which indicated the formation of B-type HA by partial substitution of CO_3^{2-} for PO_4^{3-} group.

All 18 samples prepared at $200\text{ }^\circ\text{C}$ (with varying pH and different base solutions), were confirmed to be HA via XRD (Figure 4.3) and EDX analyses (Figure 4.5, Figure 4.6 and Figure 4.7). All these 18 samples were washed with de-ionised water and XRD analysis confirmed that no NH_4NO_3 salt remained which had formed during the hydrothermal synthesis. Formation of nano crystalline ($29.7\text{ -- }80.6\text{ nm}$) HA was initially confirmed based on the calculation of crystallite size at 002 plane from XRD analysis (see Table 4.2). Literature reports show that the structural analysis of minerals such as bone, enamel, dentin and synthesised HA usually focuses on the (002) reflection at $25.8\text{ } (2\theta)$ and the reflections between 30 and 35° (112 and 300 plane) of the 2θ angle. However, resolution of peaks in the range $30\text{--}35^\circ$ (2θ) is usually poor for low-crystalline materials and hence quite difficult to distinguish them [251]. The same phenomenon was found in this study. As shown in Figure 4.3, the (112) and (300) reflections in between 30 and 35° (2θ) had low resolution and could hardly be differentiated. On the contrary, because of the resolution and high intensity of the (002) reflection, FWHM of the (002) reflection could be measured more easily and more precisely, and therefore, crystallite size and crystallinity index was measured at the (002) reflection for all samples in this study.

The sharper HA peaks and larger crystallite size (increased by $54.5\text{ -- }80.6\text{ nm}$ for all samples at plane 002, measured via XRD) were also observed for the samples prepared at higher temperature ($350\text{ }^\circ\text{C}$). This data was in a good agreement with the results obtained by Zhu *et al.* [256] who studied the nucleation and growth of HA nanocrystals by hydrothermal method and reported that the mean diameter of the HA rods increased from 29.6 to 39.8 nm with increasing temperature from 140 to $220\text{ }^\circ\text{C}$. They also reported a lower crystallinity for the

sample processed at lower temperature [256]. These results suggested that controlling the reaction temperature could be applied to produce nHA at a desired crystal size and morphology.

Most of the samples produced at 200 °C resulted in sheet morphology (37 – 331 nm), whereas formation of rod morphology (11 – 35 nm in diameter) along with sheets (for samples E(10)Am-P(8)Am 200, E(10)Am-P(10)Am 200 and E(10)S-P(10)S 200) were confirmed via FEG-SEM (Figure 4.5, Figure 4.6 and Figure 4.7) and TEM (Figure 4.9). According to Lester *et. al.* [228], the formation of HA sheets are the basic morphology obtained under ‘standard’ conditions of continuous hydrothermal synthesis. They found HA sheets had formed from the solutions of $(\text{NH}_4)_2\text{HPO}_4$ and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ at 200 °C synthesised via same method [228]. They also reported that by altering the synthesis parameters of the process, different types of morphologies can be produced [228].

In the present work, it was found that increasing pH of the upflow eggshell solution using NH_4OH and a reaction temperature of 200 °C yielded a mixture of sheets and rods (as seen from the SEM images in Figure 4.5, Figure 4.6 and Figure 4.7). This was also in good agreement with Lester *et. al.* who found that a mixture of rods and sheets formed at similar conditions of higher pH with upflow of calcium nitrate solution (using NH_4OH) and at 200 °C [228]. On the other hand, by increasing pH of the downflow diammonium hydrogen phosphate solution instead of the upflow and maintaining all other parameters, no significant morphological change was observed (see Figure 4.5, Figure 4.6 and Figure 4.7). However, these results conflicted with the findings of Ed Lester *et. al.* as they observed formation of nano rods at 200 °C with increasing pH of downflow diammonium hydrogen phosphate solution [228]. However, it is worth noting that they used calcium nitrate solution as the upflow solution.

Moreover, Lester *et. al.* [228] found that rod/tube morphologies could be produced at higher reaction temperature of 350 °C. They also reported that nanotube formation was base-specific and NH_4OH was suitable for tube formation [228]. Accordingly, four further experiments with eggshell solution and diammonium hydrogen phosphate solution were conducted at 350 °C using NH_4OH as a base solution for three experiments and NaOH for one experiment. HA was again obtained from these samples (confirmed via XRD, EDX and FTIR, see Figure 4.4, Figure 4.8 and Figure 4.12, respectively). A mixture of rod and sheet formation was confirmed via TEM analysis (Figure 4.10A and Figure 4.10B) for the samples produced by

increasing pH of either the upflow eggshell solution or of the downflow diammonium hydrogen phosphate solution.

Interestingly, at higher pH of both the upflow (eggshell solution) and downflow (diammonium hydrogen phosphate solution), predominant tube structures formed for E(10)Am-P(10)Am 350) as confirmed via TEM (Figure 4.10C).

By changing the base solution NaOH instead of NH_4OH to maintain the pH of the precursor solution, only sheet morphology formation was observed for all the samples produced (except E(10)S-P(10)S 200) at 200 °C as confirmed via FEG-SEM (Figure 4.5, Figure 4.6 and Figure 4.7). The formation of rods within the sheet morphology for E(10)S-P(10)S 200 was confirmed via FEG-SEM (Figure 4.7D1), although the TEM image of this sample was not clear (Figure 4.8B). However, predominant hexagonal HA rod formation was confirmed for this sample at higher temperature (350 °C) via FEG-SEM (Figure 4.8D2) and TEM images (Figure 4.10D).

By increasing either the reaction temperature or pH, the particles synthesised were more crystalline and the sheets became fragmented into nanorods. However, the combined effect of higher temperature with the presence of NH_4OH produced nanotubes by rolling up the sheets specifically, and preventing the fragmentation of sheets [228]. To understand the formation mechanism of the different morphologies, further analysis will be required. In this study it was observed that the morphology of nHA strongly depended on the process parameters (temperature, pH and base used to adjust pH) utilised. In addition, nanotube formation was not only pH specific, but also base specific.

The Ca/P ratios for all the samples produced was determined from EDX analysis and observed to vary from 1.43 for E8(Am)-P8 to 1.63 for E8(Am)-P10(S) which indicated the formation of non-stoichiometric HA. This could be attributed to the presence of Mg traces (confirmed via EDX) in the crystal lattice of the HA produced. Ofudje *et al.* [255] studied porcine bone-derived HA for tissue engineering applications and reported that the hydroxyapatite synthesised was non-stoichiometric with the Ca/P ratios of 1.56 and 1.88 for treated porcine bone and as-synthesised HAp at 1000 °C respectively, due to the presence of some beneficial trace elements (i.e. sodium, magnesium, potassium and silicon) which replaced Ca in the HA lattice. They also stated that the structure of non-stoichiometric HA had cation vacancies (Ca^{2+}) and anion (OH^-) vacancies which could be substituted by metals such as Na, Mg, Zn, Ag, Cu, Co

and non-metals such as F, Cl, CO_3^{2-} which could potentially enhance the biological functions of HA [255]. Therefore, nano crystalline HA with different morphologies can be prepared by simply changing the process parameters and the HA prepared using eggshells in this study, could potentially have similar properties of biological apatite. However, further analysis (such as *in vitro* cell culture, bioactivity and *in vivo* pre-clinical trials) would be required to confirm.

4.2.2 Samples prepared from calcium nitrate tetrahydrate and struvite solutions

To explore the potential use of struvite as a P precursor, some initial trials were conducted using $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ as a Ca precursor at 200 °C and 350 °C maintaining factors same as those used in the earlier section above. The pH = 6-7 and pH = 10 – 11 were maintained for struvite solutions and $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ solutions respectively, using NH_4OH and NaOH (1M). Higher pH of struvite solution could not be prepared as precipitation started just above pH 6. Solubility of struvite depends on temperature and pH [257]. Ariyanto, E. *et. al.* [257] reported that the solubility of struvite decreased with increasing pH until it reached a minimum solubility at pH 7.4. However, solubility of struvite increased at above pH=9. Moreover, they also observed that its solubility increased from 25 °C to 35 °C and decreased at 40 °C [257]. The dissolution of struvite between 25 – 35 °C requires energy (endothermic) for the solute particle to enter into solution. Increasingly solute particles in solution will increase the solubility of a solid. At above 35 °C, the dissolution of a solid releases heat and solubility decreases as the temperature increases [257]. In this study struvite solution of pH≈7 and above could not be prepared as the temperature used was 40 °C.

Pure magnesium whitlockite (Mg-WH) and/or biphasic Mg-WH and HA formed (as confirmed via XRD, EDX and FTIR analysis, see Figure 4.13, Figure 4.14 and Figure 4.16, respectively) when calcium nitrate tetrahydrate and struvite solutions were used. Mg-WH has widely been used in many fields, such as protein adsorption, drug delivery and heavy metal adsorption etc. [258].

The formation of Mg-WH in all cases was due to the presence of magnesium in the struvite. The formation of low crystalline Mg-WH or highly crystalline Mg-WH and/or biphasic Mg-WH and HA was also dependent on the Mg^{2+} concentration in the precursor solution [259]. According to the literature, magnesium inhibits the formation of apatite [248]. Substitution

of magnesium for calcium in the apatite structure would inhibit crystallisation by structural deformation of the crystal nuclei [260].

The (Ca+Mg)/P element molar ratio of all phase pure Mg-WH samples were 1.4 which was close to the standard Mg-WH powder ($\text{Ca}_{18}\text{Mg}_2(\text{HPO}_4)_2(\text{PO}_4)_{12}$) with (Ca + Mg)/P of 1.428 [258]. However, a slight increase of the (Ca+Mg)/P ratios were observed in the range of 1.42 – 1.56 for Ca(W-Am)-St(N-Am) 200 was suggested to due to the formation of another phase of HA as more Ca^{2+} present in the product suspension. Similarly, the higher (Ca+Mg)/P ratio of Ca(W-Am)-St(Ac-Am) 350 confirm the higher percentage of the HA phase formation . In addition, the Mg/Ca ratio of Mg-WH produced were in the range of 0.1-0.15 and for mixed phases in which HA was present, the ratios were 0.02 and 0.09. These results matched with the findings of Li *et.al.* [248], as they obtained the Mg/Ca ratio of 0.05 for HA and 0.12-0.15 for Mg-WH.

Moreover, it has been reported that the Mg-WH was formed when Mg^{2+} ions were present in acidic solutions containing calcium phosphate. In addition, *in vivo* formation of Mg-WH occurred under acidic conditions via the release of acidic molecules when osteoclasts resorbed old bone [180]. Jang *et. al.* [261] also confirmed that HA can directly transform into Mg-WH under proper acidic pH conditions with a sufficient amount of Mg^{2+} ions. In this study, the pH of the suspensions formed was found to be in the range of 5 – 7, which is the ideal condition for the formation of Mg-WH and/or mixture of Mg-WH and HA.

The formation of biphasic (Mg-WH and HA) and single-phase Mg-WH and morphology of crystals is also dependent on reaction temperature when NH_4OH was utilised as pH buffer and HNO_3 used to dissolve struvite. For example, at lower temperature of 200 °C almost same proportion of Mg-WH (49 %) and HA (51 %) were formed in small amount, whereas a single phase of Mg-WH formed at higher temperature (Figure 4.13A). Irregular shaped smaller particles including plates (approximately less than 30 nm) formed (as confirmed via FEG-SEM and TEM, Figure 4.14A1 and Figure 4.15A) at lower temperature (200 °C), whilst larger particles of rhombohedral shape formed (FEG-SEM and TEM, (Figure 4.14A2 and Figure 4.15B) at higher temperature (350 °C). Similar rhombohedral morphology of the Mg-WH was observed by Jang *et. al.* where whitlockite was synthesised via a precipitation method from calcium hydroxide ($\text{Ca}(\text{OH})_2$), magnesium hydroxide ($\text{Mg}(\text{OH})_2$) and phosphoric acid (H_3PO_4) in an aqueous system [262].

However, an opposite phenomenon was observed when acetic acid was used instead of HNO_3 to dissolve the struvite in this study. Semicrystalline Mg-WH formed at 200 °C and HA (88 %) with secondary phase of Mg-WH (12 %) formed at 350 °C (Figure 4.13C). Plate shaped Mg-WH formed at low temperature (200 °C) (confirmed via TEM, See Figure 4.15D). Interestingly, tube morphology of HA (50-160 nm diameter and 20-40 nm thickness) along with rhombohedral Mg-WH (23 – 720 nm) formed at higher temperature of 350 °C (confirmed via TEM, Figure 4.15E). As such, the crystallinity of the product depended on reaction temperature whereas the percentage of HA formation and Mg-WH phases and morphology of HA were strongly depended on the acids used to prepare the struvite solution.

Crystallinity of the samples prepared from $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ and struvite solutions in this second part were found to be reaction temperature dependent as an amorphous phase along with small amounts of crystalline phases (HA and Mg-WH) formed at lower temperature of 200 °C whereas, more crystalline phase pure Mg-WH formed at higher temperature of 350 °C (Figure 4.13). These results strongly correlated to the outcomes of the first section using eggshell solution and diammonium hydrogen phosphate solution where highly crystalline and larger crystallite size of HA formed at higher temperature (350 °C). Lin, C., *et. al.* also observed increasing peak intensity of Mg-WH with increasing reaction temperature from 120 to 180 °C resulting in well-crystallised particles [258] .

The crystallinity of the samples (observed from XRD pattern) also correlated to the FTIR spectra (Figure 4.16) where, increase in peak intensity observed for PO_4^{3-} group at around 560 and 601 cm^{-1} at higher temperature (350 °C) compared to lower temperature (200 °C) (Figure 4.16). Lin *et. al.* also found stronger peak signal of PO_4^{3-} at 563 and 605 cm^{-1} with increasing temperature from 120 °C to 180 °C , where Mg-WH microspheres formed from adenosine Na_2ATP , CaCl_2 and MgCl_2 [258].

The characteristic band for HA at 629 cm^{-1} and 3570 cm^{-1} assigned to OH^- mode and more pronounced FTIR bands assigned to the four PO_4^{3-} modes appeared in the range of 559 – 600 cm^{-1} and 962 – 1087 cm^{-1} for the sample $\text{Ca(W-Am)-St(Ac-Am)} 350$ (acetic acid used to dissolve struvite) which indicated formation of HA as the main phase, as identified by XRD (76.37 % HA). A reduction in the resolution of these bands for the rest of the samples indicated that HA had become secondary phase with the formation of Mg-WH as a prominent phase. A similar FTIR spectrum of biological HA was reported by Lin *et. al.*, who stated that the gradual

merger of PO_4^{3-} peaks and disappearance of OH⁻ mode and stretching bands at around 634 and 3571 cm^{-1} occurred with the formation of Mg-WH [258].

The presence of characteristic bands at 1552 - 1554 cm^{-1} associated with A-type CO_3^{2-} and the absorption bands at around 874 cm^{-1} and 1417 cm^{-1} assigned to B-type CO_3^{2-} vibration mode ν_2 and ν_3 for sample Ca(W-Am)-St(Ac-Am) 350 suggested that a certain amount of CO_3^{2-} ions were present in the both OH⁻ site and PO_4^{3-} tetrahedra within the HA structure. Similar findings for the presence of CO_3^{2-} ions in the PO_4^{3-} tetrahedra within the HA structure were reported by Lin *et. al.* [258]. They observed the bands at 1562 and 1582 cm^{-1} associated with A-type CO_3^{2-} and B-type CO_3^{2-} at 878 cm^{-1} and in the region of 1411-1450 cm^{-1} for their samples obtained [258]. As there was no CO_3^{2-} containing precursors used in this experiment, the existence of the CO_3^{2-} was most likely because of impurities contained in the starting materials as they were probably not degassed prior to use or perhaps originated from the acetic acid.

The band at 925 cm^{-1} corresponds to the vibration of the HPO_4^{2-} group present in Ca(W-Am)-St(Ac-Am) 350, where HA was the predominant phase. This meant that nonstoichiometric HPO_4^{2-} containing hydroxyapatite was formed. According to the literature, the presence of this group suggested that Mg^{2+} ions had been incorporated into the HA structure, where PO_4^{3-} groups showed a significant reduction in intensity for the Mg-WH containing samples relative to the more HA containing samples. Spread and deconvolution of the PO_4^{3-} band in the range of 991 cm^{-1} - 1059 cm^{-1} were visible for Ca(W-Am)-St(N-Am) 350, indicated the formation of Mg-WH. Moreover, appearance of low intensity bands PO_4^{3-} at 922 cm^{-1} was in good agreement with increasing Mg-WH formation and content [263].

Bands assigned for NO_3^- deformation (ν_2) at 825 cm^{-1} were only present in the case of Ca(W-Am)-St(N-Am) 350 and Ca(W-Am)-St(Ac-Am) 350, which indicated that some residue from the precursors or reactants remained. Kannan and co-workers reported that the band at 875 cm^{-1} could be assigned to the presence of nitrates and other residual species, such as NO_3^- and NH_4^+ possibly originating from precursors or reactants used during the synthesis [248].

Therefore, phase pure Mg-WH or mixtures of HA and Mg-WH could be prepared by altering the process parameters via the hydrothermal synthesis process using calcium nitrate tetrahydrate and struvite solutions and these materials could potentially be used as biomaterials specially for bone regeneration.

4.2.3 Samples prepared from Eggshell solution and Struvite solution

Hydrothermal synthesis was also conducted using eggshell solution (pH≈10-11) and struvite solution (pH≈6-7) at 200 °C and 350 °C in part 3.

The diffraction pattern of the samples prepared using HNO₃ to dissolve both precursors and NH₄OH solution to maintain pH showed the formation of an amorphous calcium phosphate phase (ACP) (as confirmed via XRD, Figure 4.17A) containing Mg (as confirmed via EDX, Figure 4.18) at 200 °C, whereas poorly crystalline peaks of Mg-WH along with HA as a secondary phase were observed at higher temperature (350 °C) (Figure 4.17A). This may have been due to the transformation of ACP to Mg-WH and HA at higher temperature [238]. The XRD results were correlated to the FEG-SEM images (Figure 4.18A2) where, formation of crystal structures of ellipsoidal and tube HA along with rhombohedral Mg-WH in amorphous phase were observed at higher temperature, which was confirmed via TEM analysis (Figure 4.19A).

On the other hand, semicrystalline homogenous Mg-WH formed at 200 °C, when NaOH was used as the pH buffer instead of NH₄OH solution. Some irregular shaped crystals within amorphous structure were also observed via FEG-SEM and TEM, (Figure 4.18B1 and Figure 4.19B). Therefore, NH₄OH also showed significant effect here on the formation of different morphologies compared to NaOH.

Interestingly, phase pure homogenous ellipsoidal shaped HA was formed at lower temperature (200 °C,) whereas a secondary phase of HA along with Mg-WH formed at higher temperature (350 °C,), when precursor solutions were dissolved using acetic acid instead of nitric acid and NH₄OH. This may be attributed to the different ionic concentrations (especially Ca²⁺ and Mg²⁺) in solution when struvite was dissolved in strong HNO₃ and weak acetic acid and the variation of pH at different temperature (200 and 350 °C). In homogeneous precipitation conditions, unstable ACP formed first, and two possible transformation approaches may then have occurred according to the pH conditions maintained [264]. When the pH ranges from 7 to 9, ACP is initially transformed into a precursor octacalcium phosphate phase and then into HA phase [264]. In contrast, when the pH is higher than 9, the intermediate conversion step is by-passed and amorphous calcium phosphate converts directly to HA [264]. As such, HA and/or amorphous calcium phosphates were co-precipitated with Mg-WH from the solutions simultaneously with calcium and Mg ions. Moreover, pH was confirmed to have an important role for the precipitation of both Mg and calcium phosphate

as both HA and Mg-WH formation were found to be pH sensitive [260, 261]. Hydroxyapatite is known as the most thermodynamically stable phase at near-neutral pH values [260] and Mg-WH is one of the most thermodynamically stable Mg-incorporated calcium phosphate compounds in acidic conditions [261]. In the present work, the final product pH for all the samples produced using NH_4OH was about 9 and pH ~ 5 observed while NaOH was used as a pH buffer. These values justified the formation of HA as the predominant phase and phase pure Mg-WH in the case of using NH_4OH and NaOH, respectively.

These XRD results also correlated to the FTIR analyses (Figure 4.20), where higher resolution peaks of PO_4^{3-} were observed when acetic acid was used to dissolve the precursors resulting in higher crystalline phase formation. In the case of using nitric acid instead, peaks of PO_4^{3-} were observed to have merged into a broad peaks at around $500\text{-}600\text{ cm}^{-1}$ and $900\text{-}1100\text{ cm}^{-1}$, especially for the amorphous phase. Moreover, the observation of the characteristic OH $^-$ peak at 619 and 3572 cm^{-1} only for E(Ac-Am)-St(Ac-Am) 350 indicated the formation of highly crystalline HA. On the other hand, the observation of HPO_4^- group only for E(N-S)-St(N-S)200 indicated the formation of only Mg-WH.

Therefore, useful products of phase pure HA and Mg-WH or mixtures of HA and Mg-WH could be prepared from waste resources using eggshells and struvite by altering the parameters of the hydrothermal synthesis process. These materials could be used for bone regeneration as Mg-WH also present in the natural bone. In a recent study, Mg-WH showed better osteogenic activity and increased bone formation than HA by the continuous release of magnesium and phosphate ions [265]. Moreover, the presence of both HA and Mg-WH at approximate 1:3 ratio promotes the osteogenic activity [180]. However, further *in vitro* and pre-clinical *in vivo* trials would be required to confirm the efficacy of these materials.

Chapter 5

Sustainable production of calcium phosphates (CaPs) via ball milling and flame spheroidisation

5.1 Introduction

The significance of the environmentally friendly design of materials and methods has growing awareness in recent years. The sustainability perception is greatly affecting the chemical community and they are focusing more on reducing hazardous ingredients utilisation by implementing green strategies from renewable, sustainable resources as starting materials. To address this issue, ball milling, being a simple mechanochemical method is gaining attention in chemistry now-a-day [27, 266]. This method does not utilise any chemical solution and provides high reproducibility with low production expense and also offers high manufacturing capabilities on industrial-scale [266]. A variety of functional nanocrystalline and amorphous materials can be produced by this method, for which a further heat treatment of the samples may require depending on the applications. Highly crystalline pure, biphasic or multiphasic CaPs can be obtained which depends on the type and composition of the materials and the temperature [29]. The CaPs possess bioactivity, biocompatibility and biosorption characteristics and thus they are great candidates to be used as bone graft substitutes for bone defect treatment, hard tissue engineering, local biological factors delivery and reinforcing of composites for soft tissue engineering and load-bearing applications [26].

Among the different forms of bone substitutes, microspheres, having higher flow properties, larger surface area, enhanced packing densities and higher ion and degradation rate [31], exhibits more advantages in treating complex-shaped bone defects, especially in comparison to irregularly-shaped particles and nanofillers [267]. In addition, they can be transferred to the particular target site via minimally invasive route [31]. Therefore, microspheres are extensively studied in the biomedical field to be used in drug delivery, bone tissue engineering and regeneration, absorption and desorption of substances etc. [30]. Calcium phosphate (CaP) microspheres provide excellent biocompatibility, osteoconductivity and higher mechanical properties compared to those of other particles geometries and thus can be used

in the orthopaedics, dentistry and the pharmaceutical sectors. Several methods are applied to manufacture microspheres, such as precipitation, spray pyrolysis, electrospraying, emulsification processes, flame spheroidisation, etc. The flame spheroidisation process is very fast, simple and inexpensive [30].

The aim of the work in this chapter was to explore another sustainable route ball milling followed by sintering and/or flame spheroidisation to prepare HA and/or other CaPs particles /microspheres from natural waste resources (such as eggshells and struvite) for biomedical applications. The precursor materials were first milled in a planetary ball mill and then ball-milled samples were sintered at 700, 900 and 1150 °C or processed via flame spheroidisation and their characterisation was conducted via XRD, TGA/DSC, FEG-SEM, EDX and FTIR.

5.2 Results

This section represents the results of the samples produced [hydroxyapatite (HA)/Calcium phosphates (CaPs)] via Ball milling method followed by sintering and flame spheroidisation process using raw eggshells and calcined eggshells as a Ca precursor and struvite as a P precursor. The outcomes have been reported in two experimental parts. In the first part, ball milled samples were sintered at different temperatures (i.e. 700, 900 and 1150 °C) and outcomes of the flame spheroidisation process of ball milled samples is reported in the second part. The calcination of eggshells was conducted at 700 and 900 °C to obtain CaO. The experimental parameters for calcination of eggshells and for the preparation of HA/CaPs via ball milling followed by sintering and flame spheroidisation processes and their characterisation techniques are highlighted in Figure 5.1.

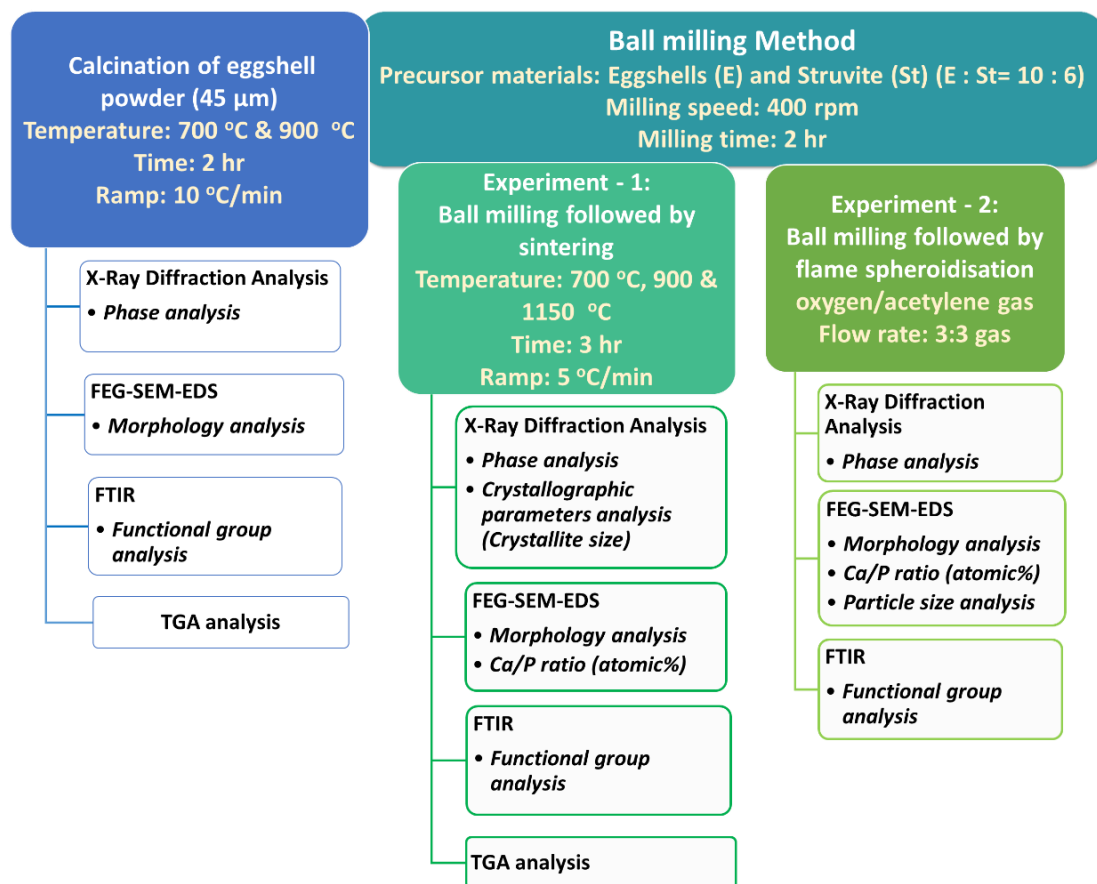


Figure 5.1: Schematic flow charts of experimental sections including process parameters and characterisation techniques used for the calcination of eggshells and for the samples obtained via ballmilling followed by sintering and flame spheroidisation using raw/ calcined eggshell powder and struvite.

5.2.1 Calcination of Eggshells

5.2.1.1 X-ray diffraction analysis

Figure 5.2 shows the XRD pattern of the starting raw eggshell powder and sintered eggshell powder at 700 and 900 °C. The XRD pattern for the pre-sintered eggshell powder and eggshell powder sintered at 700 °C were a match for CaCO_3 spectra (ICDD no. 00-017-0763). Whereas, for the eggshell powder sintered at 900 °C for 2hrs most of the CaCO_3 peaks had disappeared and only CaO peaks (ICDD no. 00-004-0777) were observed.

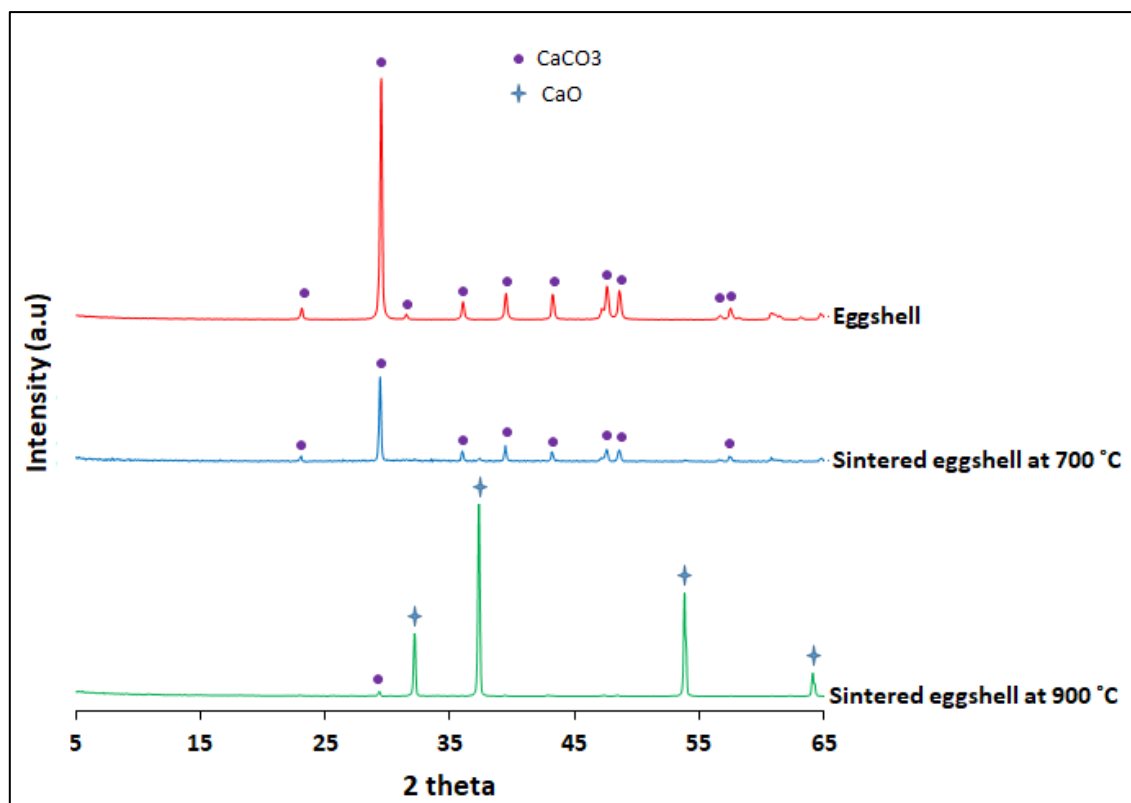


Figure 5.2: The XRD trace of raw eggshell powder, eggshell powder sintered at 700 °C and eggshell powder sintered at 900 °C.

5.2.1.2 Scanning electron microscopy

Figure 5.3 A-C show scanning electron micrographs of the surface of raw eggshell powder and eggshell powder sintered at 700 and 900 °C, respectively. Small changes in surface morphology can be observed after sintering the eggshells at 700 °C. However, a significant change in surface morphology was observed for eggshell powder sintered at 900 °C in comparison (see Figure 5.3 C).

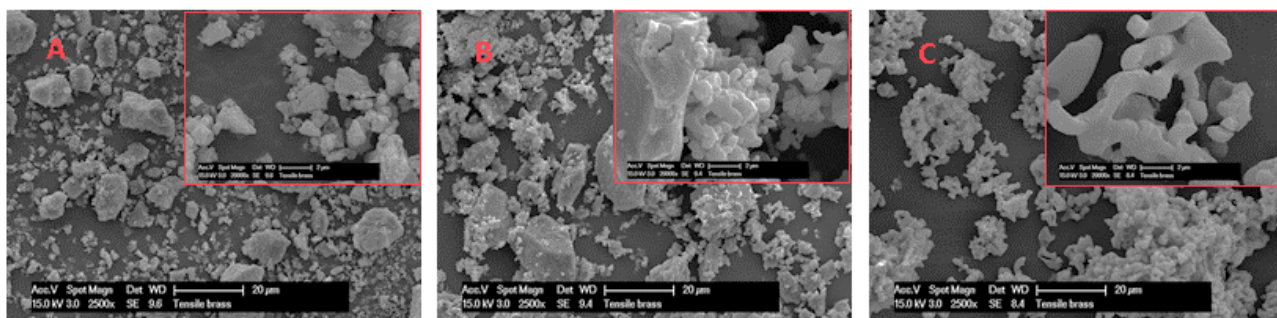


Figure 5.3: Surface morphology of A) raw eggshell powder, B) eggshell powder sintered at 700 °C and C) eggshell powder sintered at 900 °C.

5.2.1.3 FTIR analysis

Figure 5.4 shows the FTIR spectra of raw eggshell powder and eggshell powder sintered at 700 and 900 °C for 2hrs. The figure reveals that the most significant peak of intensity was observed for the raw eggshell powder at around 1406 cm^{-1} which associated with the C–O stretching of calcium carbonate present within the sample matrix [268]. The other two well-defined peaks at 873 and 711 cm^{-1} , were correlated to C–O bending modes and Ca–O bonds of calcium mineral, respectively [268]. FTIR spectrum of eggshell powder sintered at 700 °C exhibited same result as raw eggshell powder. However, when eggshell powder was sintered at 900 °C, the peak intensity at the same three positions mentioned above, significantly decreased and an additional peak at around 3640 cm^{-1} was observed which associated with the presence of -OH group in Ca(OH)_2 due to the adsorption of water by CaO. The wide and strong band at around 500 cm^{-1} corresponds to the Ca–O band [268].

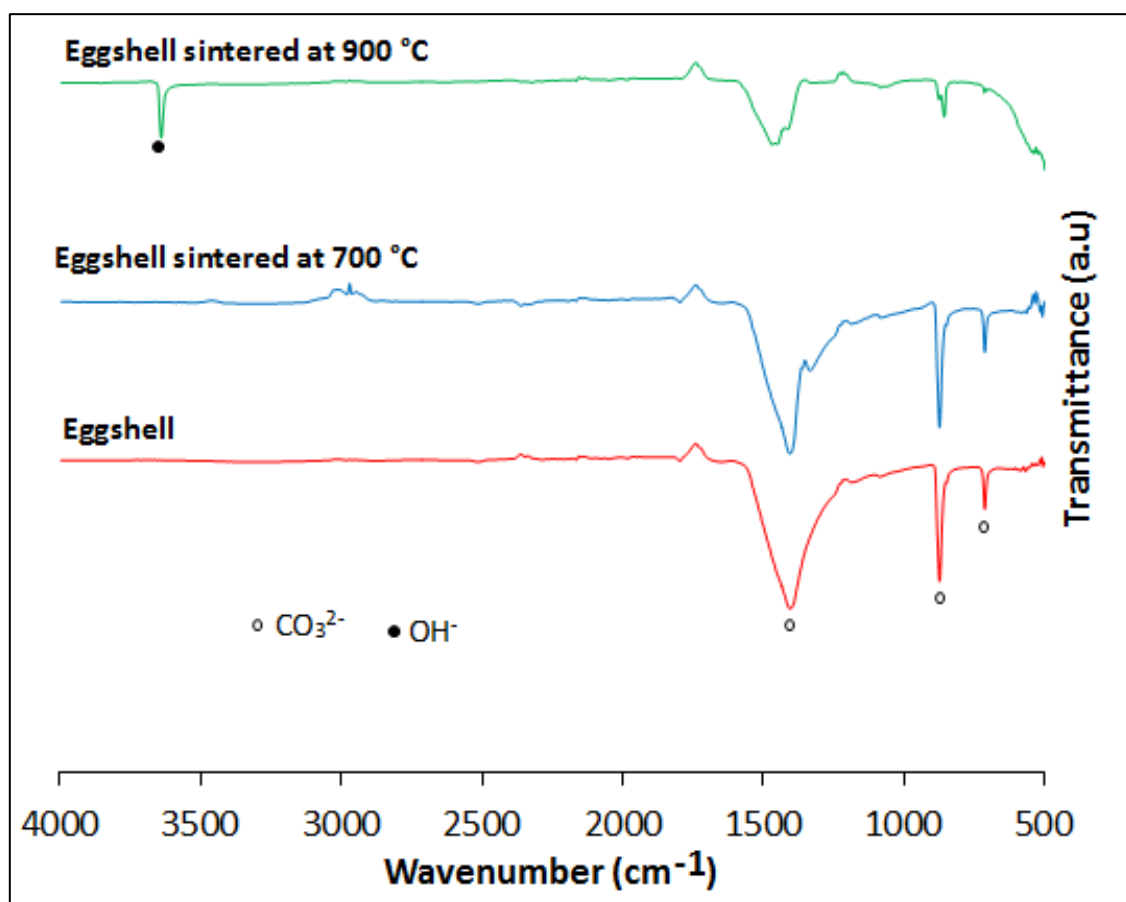


Figure 5.4: FTIR spectra of raw eggshell powder and eggshell powder sintered at 700 and 900 °C for 2 hrs.

5.2.1.4 Thermogravimetric Analysis (TGA)

Figure 5.5 shows the TGA analysis of raw eggshell powder and sintered (900 °C) eggshell powder. From the TGA curves, around 1 % weight loss for raw eggshell powder was observed, whereas no significant change in wt % loss was observed for the sintered eggshell powder from room temperature to 200 °C. A further weight loss of 2.2 % for raw eggshell powder was observed when the temperature was increased to 600 °C. The prominent weight loss of approximately 42.8 % for raw eggshell powder was seen over the temperature range of 600 – 780 °C. On the other hand, the sintered eggshell powder revealed an approximate 2% and 1% weight loss when heated to 400 °C and 600 °C, respectively. An approximate 4% weight loss was observed for sintered eggshell powder when the sample was heated to 1200 °C.

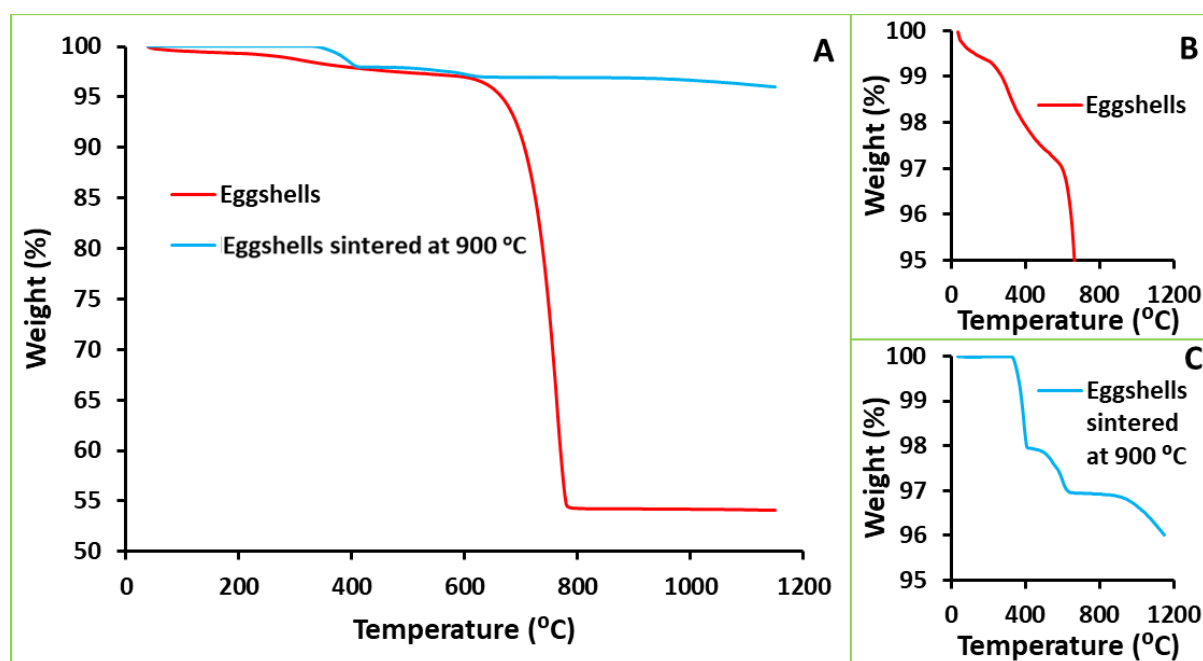


Figure 5.5: A) combined representation of TGA analysis for raw eggshell powder and eggshell powder sintered at 900 °C for 2 hrs and TGA analysis for individual B) raw eggshell powder and C) sintered eggshell powder with smaller scale range (95-100) of Y axis. A) combined representation of TGA analysis for raw eggshell powder and eggshell powder sintered at 900 °C for 2 hrs and TGA analysis for individual B) raw eggshell powder and C) sintered eggshell powder with smaller scale range (95-100) of Y axis.

5.2.2 Calcium phosphates (CaPs) synthesis via ball milling followed by sintering

Raw eggshells (E)/calcined eggshells (Ecal) and struvite were ball milled for 2 hrs at 400 rpm and the samples produced were sintered at 700, 900 and 1150 °C for 3 hrs with ramp rate of 5 °C/min and their characterisations were conducted via XRD, SEM, EDX, FTIR and TGA.

5.2.2.1 Investigating phase changes using XRD

Figure 5.6 shows the X-ray diffraction patterns of the samples synthesised by milling struvite with raw eggshell (Figure 5.6A) or calcined eggshell powders (Figure 5.6B) for 2 hr followed by subsequent sintering at 700, 900 and 1150 °C for 3 h.

As can be seen from the diffraction pattern of ball milled powder (without sintering), the peaks corresponded to the unreacted precursors, i.e, struvite and CaCO_3 .

The XRD pattern of the samples sintered at 700 °C matched with HA (ICDD 00-009-0432), Farringtonite ($\text{Mg}_3(\text{PO}_4)_2$) (ICDD 00-033-0876), Mg-Whitlockite ($\text{Ca}_{18}\text{Mg}_2(\text{HPO}_4)_2(\text{PO}_4)_{12}$) Mg-WH (ICDD 01-070-2064) and Stanfieldite ($\text{Mg}_3\text{Ca}_3(\text{PO}_4)_4$) (ICDD 01-073-1182). The peaks of CaCO_3 (ICDD 01-083-1762) from unreacted precursors was also found in this stage. Predominant peaks of Stanfieldite with small amounts of HA and Mg-WH peaks were observed when the samples sintered at 900 °C. Trace amounts of MgO (ICDD 00-004-0829), CaCO_3 and CaO (ICDD 00-048-1467) were also observed at this temperature. However, the predominant phase of Mg-WH along with HA and MgO peaks were matched for the sample sintered at higher temperature of 1150 °C.

On the other hand samples produced from calcined eggshells and struvite via ball milling were found to have formed HA mainly with a small amount of brucite ($\text{Mg}(\text{OH})_2$) as shown in Figure 5.6B. There were no peaks corresponding to the precursor materials or CaO observed. By sintering of this material at 700 °C, prominent phase of HA peaks and small amount of MgO peaks were found. With a further increase in sintering temperature to 900 °C, the XRD pattern were matched with HA, Mg-WH along with MgO. Any other phase transformations could not be detected with increasing temperature to 1150 °C. High intensity peaks of HA, Mg-WH and small amount of MgO were observed at 1150 °C as seen in the XRD pattern in Figure 5.6B.

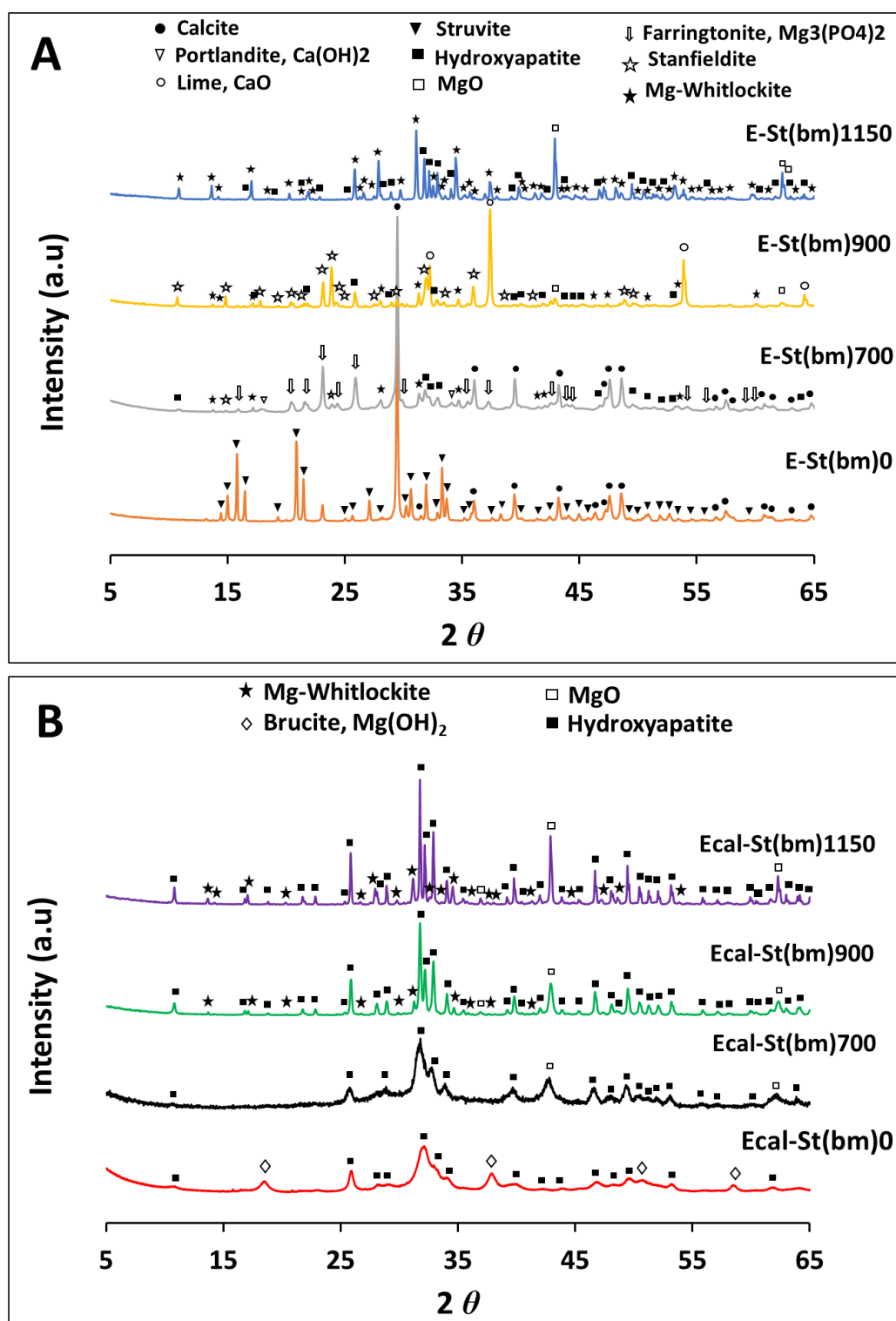


Figure 5.6: XRD pattern of samples synthesised via ball milling and followed by sintering at 700 °C, 900 °C and 1150 °C using (A) raw eggshells (E) and struvite (St), and (B) calcined eggshells (Ecal) and struvite.

5.2.2.2 Crystallographic parameters (Crystallite size)

Crystal sizes of the calcium-based phosphates produced at different temperatures (i.e., 0, 700, 900 and 1150 °C) using ball milled raw/calcined eggshell powder and struvite are presented in Table 5.1. The crystallite size of HA and Mg-WH phases had mainly been considered to evaluate the variation of crystallite size at different sintering temperature. The crystallite size of HA and Mg-WH produced was responsible for Bragg reflections of the (002) and (0210) planes (respectively) which were calculated from XRD data using the Scherrer equation [94]. The crystallite size of HA increased from 53 to 80 nm for the samples prepared from raw eggshells and struvite and increased from 30 to 80 nm for the samples prepared from calcined eggshells and struvite with increasing the sintering temperature from 700 to 1150 °C. Whereas, the crystallite size of Mg-WH remained almost constant (around 30 nm) for both samples even with the variation of sintering temperature. The overall percentage of crystallinity of the samples produced from raw eggshells and struvite increased from 67% (for E-St700) to 90% (for E-St1150) with increasing the sintering temperature from 700 to 1150 °C. Whereas, for using the calcined eggshells and struvite, the crystallinity increased from 46% to 88% with increasing the sintering temperature from 700 to 1150 °C.

Table 5.1: Crystallite size at (002) and (0210) plane for HA and Mg-WH, respectively and overall crystallinity of samples prepared from raw/calcined eggshell powder and struvite via ballmilling followed by sintering at 700, 900 and 1150 °C.

Samples name	Crystallite size (nm)		Crystallinity (using auto background correction)
	At plane (002) for HA	At plane (0210) for Mg-WH	
E-St(bm)0	-	-	89
E-St(bm)700	53	28	68
E-St(bm)900	81	82	88
E-St(bm)1150	81	33	90
Ecal-St(bm) 0	81	-	67
Ecal-St(bm) 700	30	-	46
Ecal-St(bm) 900	81	30	87
Ecal-St(bm) 1150	81	29	88

5.2.2.3 Morphology characterisation using FEG-SEM-EDS and TEM

Figure 5.7 shows the SEM images of the samples synthesised from raw eggshells-struvite powders (Figure 5.7A) and calcined eggshells-struvite powders (Figure 5.7B) via ball milling followed by sintering at 0 °C (without sintering), 700 °C , 900 °C and 1150 °C. As seen from these images without sintering the agglomerated powders were irregular shaped particles of nano to submicron size range. Upon sintering (from 700 to 1150 °C), the agglomeration of the particles became more prominent, as can be seen from Figure 5.7.

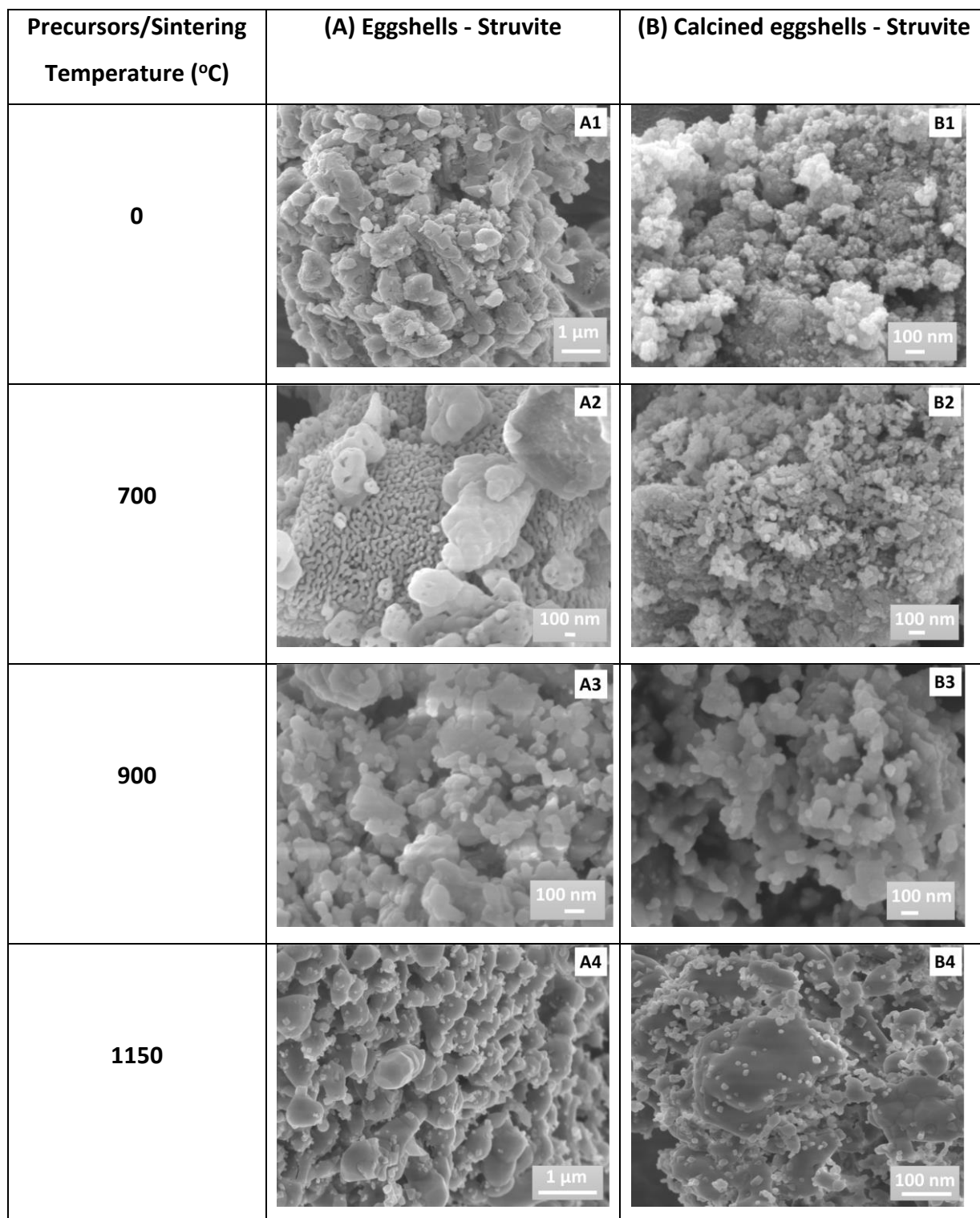


Figure 5.7: Surface morphology of samples synthesised via ball milling followed by sintering at 0 °C (without sintering), 700 °C, 900 °C and 1150 °C using (A) raw eggshells and struvite, and (B) calcined eggshells and struvite.

The Ca/P ratios of the samples synthesised from raw eggshells - struvite, and calcined eggshells - struvite using ball milling followed by sintering at 0 °C (without sintering), 700 °C, 900 °C and 1150 °C are presented in Table 5.2. The Ca/P ratio (atomic %) was found to be 1.5-1.67 for the samples prepared from raw eggshells and struvite and the variation of Ca/P ratio was found to be random with increasing sintering temperature from 0 to 1150 °C. However, the Ca/P ratio was observed to have increased gradually from 1.5 to 1.6 with increasing sintering temperature from 0 to 1150 °C for the samples prepared from calcined eggshells and struvite.

The range of (Ca+Mg)/P ratio (atomic %) was observed to be between 1.68 – 2.97 for the samples produced using raw eggshells and struvite. An increasing trend from 1.53 to 2.82 of the ratios of (Ca+Mg)/P was seen with increasing the sintering temperatures (from 0 to 1150 °C) for the samples produced from calcined eggshell powder and struvite.

Table 5.2: Ca/P and (Ca+Mg)/P ratio (atomic%) of samples synthesised via ball milling followed by sintering at 0 °C (without sintering), 700 °C, 900 °C and 1150 °C using (A) raw eggshells and struvite, and (B) calcined eggshells and struvite.

Precursors	Sample no.	Sample name	Average Ca/P (atomic %)	Average Ca+Mg/P (atomic %)
Eggshells (E) and Struvite (St)	A1	E-St(bm)0	1.67 ± 1.74	2.88 ± 2.01
	A2	E-St(bm)700	1.58 ± 0.12	1.68 ± 0.1
	A3	E-St(bm)900	1.63 ± 0.01	2.97 ± 0.01
	A4	E-St(bm)1150	1.50 ± 0.01	1.95 ± 0.02
Calcined Eggshells (Ecal) and Struvite (St)	B1	Ecal-St(bm)0	1.52 ± 0.05	1.53 ± 0.08
	B2	Ecal-St(bm)700	1.53 ± 0.08	2.66 ± 0.12
	B3	Ecal-St(bm)900	1.60 ± 0.20	2.80 ± 0.13
	B4	Ecal-St(bm)1150	1.60 ± 0.03	2.82 ± 0.07

5.2.2.4 Functional group analysis by FTIR

To study the evolution of functional groups in the ball milled powder samples and those sintered at different temperatures, FTIR analysis of these samples was analysed, as presented in Figure 5.8. The band positions and their respective assignments are given in Table 5.3. The FTIR spectra of pre-sintered samples produced from ballmilled raw eggshells-struvite powder (Figure 5.8A) showed noticeable bands at 874 and 1425 cm^{-1} along with a weak peak at 1796 cm^{-1} correspond to C=O from carbonate ion (CO_3^{2-}) and weak peaks at 712 and 569 cm^{-1} related to Ca-O bond, which confirmed the presence of CaCO_3 [268, 269]. Another significant band at 997 cm^{-1} corresponds to PO_4^{3-} ion present in struvite was observed [263]. In addition, the observed broad band at around 2889 cm^{-1} was due to NH_4^+ ion present in struvite or the C=O from carbonate ion [244, 269]. The weak broad bands at around 1668 and 3230 cm^{-1} assigned to N-H bending and N-H stretching vibrations, respectively and 2328 cm^{-1} of CaCO_3 were also observed for the ball milled pre-sintered samples [269, 270].

FTIR spectrum of sintered ballmilled eggshells-struvite powder at 700 °C exhibited intense peak at 1032 cm^{-1} along with 982 cm^{-1} correspond to PO_4^{3-} mode of ν_3 , ν_1 , respectively and weak bands of C=O from carbonate ion CO_3^{2-} were observed at 874, 1416 and 1796 cm^{-1} [95, 239, 242, 243, 245]. Peak at 712 cm^{-1} related to Ca–O bonds of calcium mineral also presented in this sample [268, 269]. Split peaks were observed at 579 and 599 cm^{-1} which correspond to PO_4^{3-} bending (ν_4) [271]. The characteristic band of apatite at 623 cm^{-1} related to structural OH^- was also appeared after sintering the ballmilled samples [242, 243].

Further subsequent sintered at 900 °C and 1150 °C, the characteristic peaks of apatite in the range of 986 – 1120 cm^{-1} and 550-605 cm^{-1} correspond to PO_4^{3-} had become prominent for both sintered samples [271]. However, sharp peaks of structural OH^- at 628 and 3640 cm^{-1} were not observed for the samples sintered at 900 °C, while they appeared sharply for samples sintered at 1150 °C [242, 243] [249] [95, 239, 242, 243, 245]. The decrease of bands (compared to pre-sintered samples) at 1412 cm^{-1} corresponding to CO_3^{2-} were clearly observed, whilst a sharp peak which occurred at 877 cm^{-1} (for pre-sintered samples) disappeared with the increase of sintering temperature [95, 239, 242, 243, 245].

FTIR spectra of samples produced using calcined eggshells and struvite (Figure 5.8B) showed an intense peak at 1029 cm^{-1} and a doublet at 563 and 601 cm^{-1} corresponding to PO_4^{3-} bending mode ν_3 and ν_4 , respectively which are the characteristic peaks of HA [95, 243] [271]. The bands of CO_3^{2-} bending (ν_3) at 874 cm^{-1} and a doublet at 1419 and 1466 cm^{-1} revealed the b-type substitution at PO_4^{3-} site [95, 239, 242, 243, 245]. An exceedingly small peak at 3595 cm^{-1} related to structural OH^- also indicated the formation of HA [271]. Weak broad bands at around 1635 cm^{-1} and $3400\text{--}3500\text{ cm}^{-1}$ correspond to the absorbed water along with a sharp peak at 3696 cm^{-1} of the stretching modes of OH^- bond indicated the presence of $\text{Mg}(\text{OH})_2$ in the pre-sintered ballmilled samples of calcined eggshells and struvite [243].

Upon sintering at $700\text{ }^\circ\text{C}$, intense peak of HA at 1028 cm^{-1} along with two additional peaks at 962 and 1090 cm^{-1} corresponds to PO_4^{3-} bending (ν_3), PO_4^{3-} bending (ν_1) and PO_4^{3-} stretching (ν_3) respectively were observed [95, 243, 272, 273]. Another sharp peak was present in this sample at 563 cm^{-1} along with 600 cm^{-1} related to the PO_4^{3-} bending (ν_4) [271]. Moreover, two weak bands at 3572 and 629 cm^{-1} corresponding to the structural OH^- of HA were also observed [242, 243]. Bands for CO_3^{2-} bending (ν_3) at 1416 and 1454 cm^{-1} and CO_3^{2-} bending (ν_2) at $871\text{--}873\text{ cm}^{-1}$ revealed the presence of CO_3^{2-} of b-type substitution in the HA structure [95, 239, 242, 243, 245].

The FTIR spectra of further sintered ballmilled samples at $900\text{ }^\circ\text{C}$ showed PO_4^{3-} peaks of high intensity in the range of $900\text{--}1100\text{ cm}^{-1}$ (PO_4^{3-} bending (ν_1) at 964 cm^{-1} , PO_4^{3-} bending (ν_3) at 1028 cm^{-1} , PO_4^{3-} stretching (ν_3) at 1088 cm^{-1}) and $550\text{--}600\text{ cm}^{-1}$ (PO_4^{3-} bending (ν_4) at 561 and 598 cm^{-1}) [240, 241, 248, 249]. However, band at 1088 cm^{-1} also corresponds to the vibration of the HPO_4^{2-} (non-stoichiometric) group indicated the presence of Mg-WH in the sample [95, 243, 250, 274]. The characteristic band of structural OH^- at 628 and 3572 cm^{-1} were also observed [242, 243].

No significant differences in FTIR spectra were observed for the samples (from calcined eggshells and struvite) sintered at higher temperature of 1150 compared to $900\text{ }^\circ\text{C}$.

Table 5.3: FTIR band positions and their corresponding assignments of samples produced via ball milling followed by sintering at 700, 900 and 1150 °C using raw/calcined eggshells and struvite powder.

FTIR band positions								Corresponding assignments	References
E-St(bm)0	E-St(bm)700	E-St(bm)900	E-St(bm)1150	Ecal-St(bm)0	Ecal-St(bm)700	Ecal-St(bm)900	Ecal-St(bm)1150		
569	-	-	-	-	-	-	-	metal-oxygen bond	[270]
-	579	560 - 590	571, 600	563, 601	563, 600	561, 598	569, 598	PO ₄ ³⁻ bending (ν_4)	[240, 241, 248, 249]
-	623	628	629	628	629	628	632	Structural OH ⁻	[242, 243]
712	712	-	-	-	-	-	-	Ca–O bonds of calcium mineral	[268, 269]
874	874	877	-	874	871-873	-	-	CO ₃ ²⁻ bending (ν_3) / HPO ₄ ²⁻ group / C–O bending modes bonds of calcium mineral	[95, 239, 242, 243, 245, 248, 249, 252]
		940	947		962	964	962	PO ₄ ³⁻ bending (ν_1)	[95, 243, 272, 273]
997	982	986	972	-	-	-	-	PO ₄ ³⁻ stretching (ν_1)	[95, 243, 248-250, 263]
-	-	1064	1024	1029	1028	1028	1026	PO ₄ ³⁻ bending (ν_3)	[95, 243, 250, 274]
-	-	1099, 1120	1088		1090	1088	1088	The vibration of the HPO ₄ ²⁻ group	[263, 271, 272]

1425, 2328	1416	1412	1411	1419, 1466	1416, 1454	-	-	CO ₃ ²⁻ bending (ν_3)	[95, 239, 242, 243, 245, 269]
1 668	-	-	-	-	-	-	-	N-H bending	[270]
-	-	-	-	1635	-	-	-	stretching modes of OH ⁻ (adsorbed water)	[272]
1796	1796	-	-	-	-	-	-	C=O from carbonate ion	[269]
2889	-	-	-	-	-	-	-	(2935) NH ₄ ⁺ asymmetric deformation (ν_4')/ C=O from carbonate ion	[244, 270]
3230	-	-	-	-	-	-	-	O-H and N-H stretching	[270]
-	-	-	-	3400-3500	-	-	-	Absorbed water	[249]
-	-	3640	3643	3597	3572	3572	3572	Structural OH ⁻	[249]
-	-	-	-	3696	-	-	-	Mg(OH) ₂ /Ca(OH) ₂	[243]

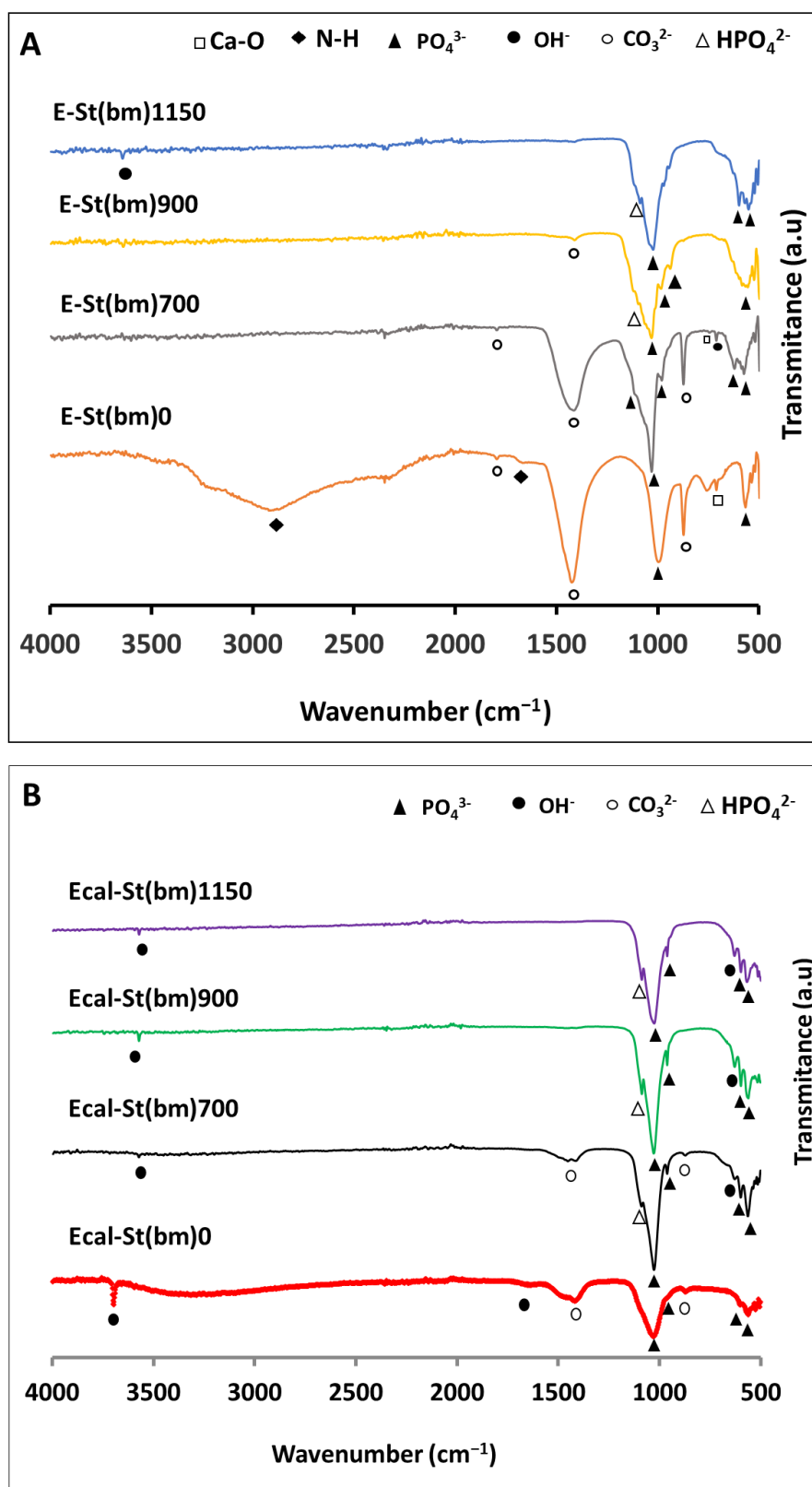


Figure 5.8: FTIR spectra of samples synthesised via ball milling and followed by sintering at 700 °C, 900 °C and 1150 °C using (A) raw eggshells (E) and struvite (St), and (B) calcined eggshells (Ecal) and struvite.

5.2.2.5 TGA analysis

The thermal analysis of ball milled samples produced using raw eggshells/calcined eggshells and struvite [i.e. E-St(bm) and Ecal-St(bm)] were analysed via thermogravimetric and differential scanning calorimetric analyses which are shown in Figure 5.9A and B, respectively. Around 25 % weight loss for E-St(bm), and 10 % weight loss for Ecal-St(bm) was observed from room temperature to 200 °C. A further weight loss of 5 % for E-St(bm) was observed when the temperature was increased to 600 °C. The prominent weight loss of approximately 17 % for the same sample was seen over the temperature range of 600 – 750 °C.

On the other hand, the Ecal-St(bm) revealed that an approximately 5.5% weight loss occurred when the samples were heated from 200 to 400 °C. Approximately, 6.5% weight loss was observed for the same sample when heated from 400 to 1200 °C.

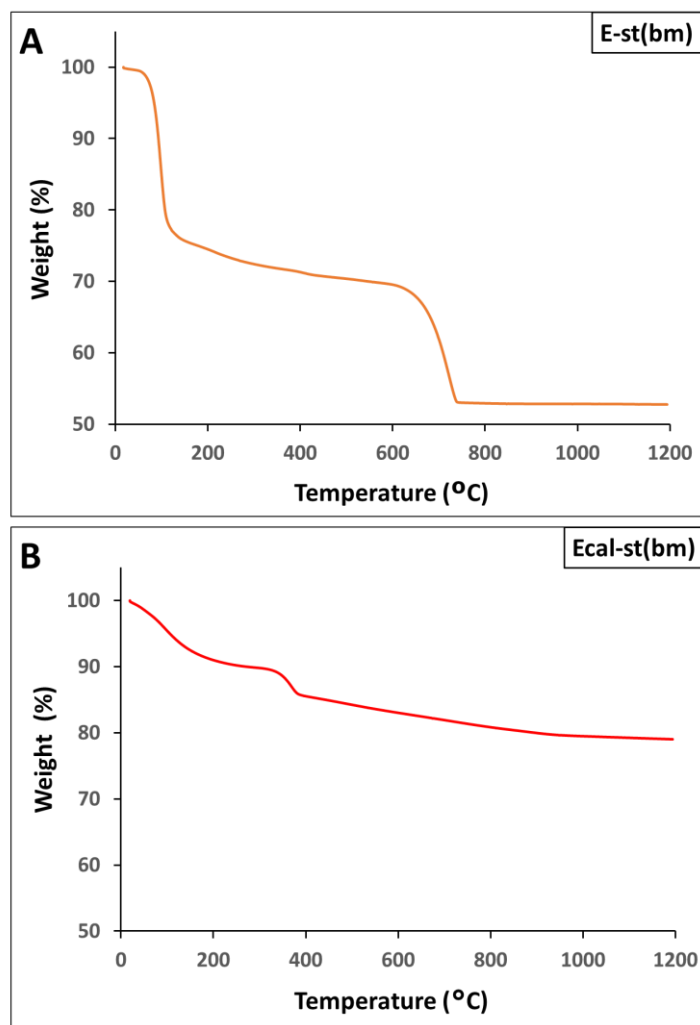


Figure 5.9: TGA of samples synthesised via ball milling using (A) raw eggshells (E) and struvite (St), and (B) calcined eggshells (Ecal) and struvite.

5.2.3 Hydroxyapatite (HA)/ Calcium phosphates (CaPs) synthesis via ball milling followed by flame spheroidisation

The flame spheroidisation process was conducted for the samples obtained via ball milling from raw eggshells/calcined eggshells and struvite.

Two different size ranges (i.e. 63 -100 μm and 100 – 160 μm) of granules (obtained via grinding using mortar and pestle of dried agglomerated ball milled particles followed by sieving) of ball milled samples were processed via flame spheroidisation and their morphological and phase changes were characterised via FEG-SEM, EDX, XRD and FTIR.

5.2.3.1 Morphology characterisation using FEG-SEM-EDX

FEG-SEM images of the products obtained from the granules of two size ranges (63 -100 μm and 100 – 160 μm) of ball milled samples (from raw/calcined eggshells and struvite) are presented in Figure 5.10. Interestingly, the starting irregular shaped particles (Figure 5.7A1 and B1 at 0 °C) were transformed into microspheres. The yield in terms of sphericity was found be more than 98% for the smaller starting granules (63 -100 μm) whereas, some irregular shaped particles were seen along with microspheres in the case of the larger granules (100-160 μm).

The Ca/P and (Ca+Mg)/P ratio (atomic%) of the samples obtained via EDX are presented in Table 5.4, indicating the Ca/P ratio was around 1.5 for sample E-St(fs)63-100 and 1.6-1.65 was found for the rest of the samples. Moreover, the (Ca+Mg)/P ratio was found to be 3 for the samples prepared (both size ranges) from raw eggshells and struvite, whereas 3.2 was found for the samples obtained from calcined eggshells and struvite.

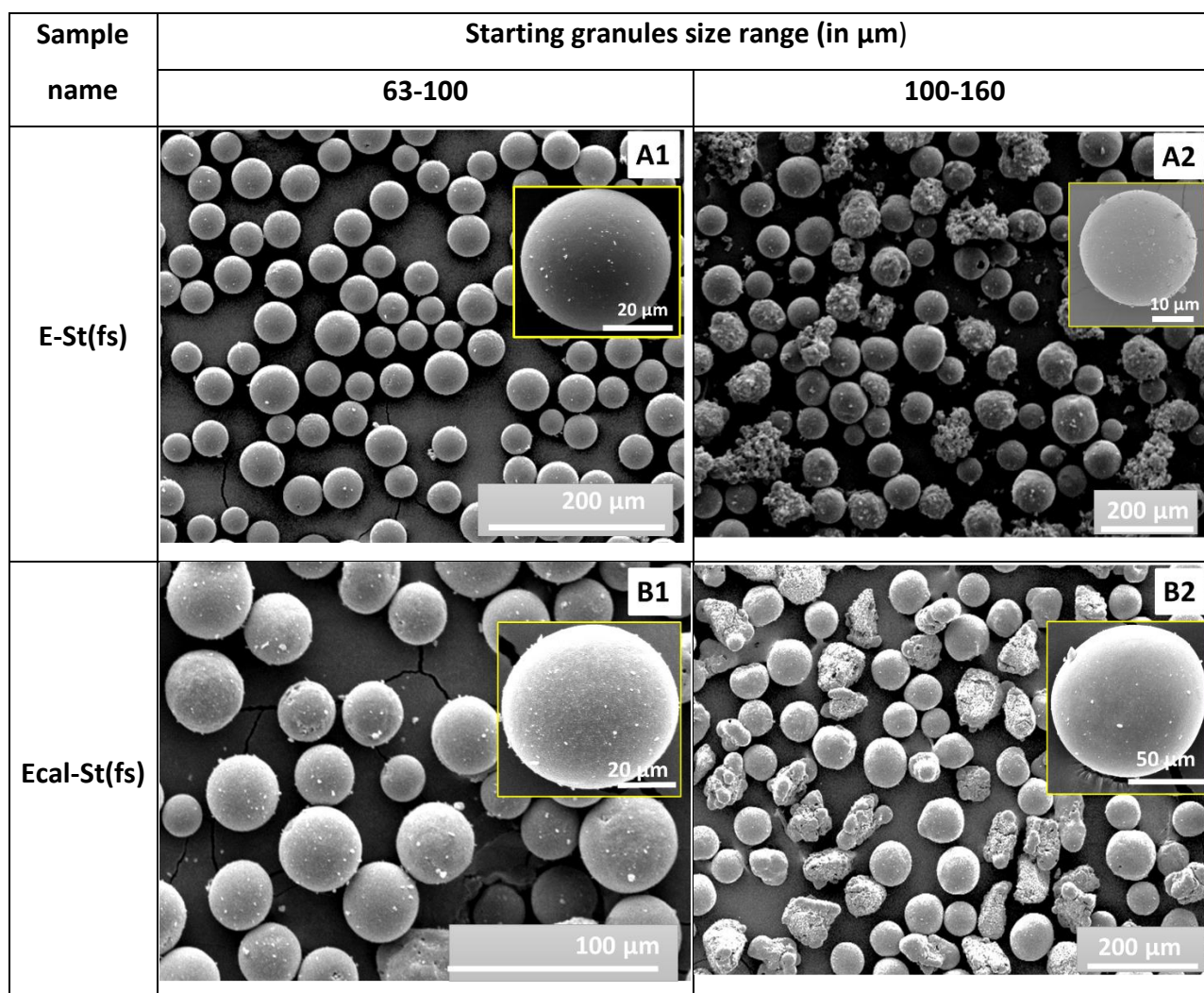


Figure 5.10: Surface morphology and Ca/P ratio (atomic) of samples synthesised via ball milling followed by flame spheroidisation using (A) raw eggshells and struvite, and (B) calcined eggshells and struvite of two size ranges of starting granules: (A1, B1) 63 – 100 μm and (A2, B2) 100 – 160 μm . MS represents for microspheres.

Table 5.4: The Ca/P and (Ca+Mg)/P ratio (atomic) of samples synthesised via ball milling followed by flame spheroidisation using (A) raw eggshells and struvite, and (B) calcined eggshells and struvite of two size ranges of starting granules: (A1, B1) 63 – 100 μm and (A2, B2) 100 – 160 μm .

Precursors	Sample no.	Sample name	Ca/P (atomic %)	Ca+Mg/P (atomic %)
Eggshells (E) and Struvite (St)	A1	E-St (fs)63-100	1.5 ± 0.12	3.00 ± 0.38
	A2	E-St (fs)100-160	1.65 ± 0.6	3.01 ± 0.47
Calcined Eggshells (Ecal) and Struvite (St)	B1	Ecal-St(fs)63-100	1.6 ± 0.08	3.20 ± 0.40
	B2	Ecal-St(fs)100-160	1.61 ± 0.11	3.24 ± 0.41

5.2.3.2 Particle Size analysis from FEG-SEM images

Particle sizes of the microspheres produced are presented in Table 5.5. Microspheres with the size ranges of 25 – 60 μm formed when smaller size range of granules (63 – 100 μm) were processed, whereas 40 – 130 μm microspheres were found to be formed for larger starting granules (100 – 160 μm).

Table 5.5: Particle size range of microspheres prepared via ballmilling followed by flame spheroidisation process from raw and calcined eggshell powder in combination with struvite in two different size ranges of 63 – 100 μm and 100 – 160 μm .

Sample name	Size range (μm)			
	Starting	Obtained	Starting	Obtained
E-St(fs)	63-100	29.8 – 54.55 (Avg. 43.87 ± 6)	100-160	42.1 – 107.64 (Avg. 72.53 ± 14.1)
Ecal-St(fs)		24.54 – 58.63 (Avg. 39.47 ± 8)		43.46 – 129.84 (Avg. 96.56 ± 15.22)

5.2.3.3 Investigating phase changes using XRD

Figure 5.11 shows the X-ray diffractogram of the samples obtained via ball milling followed by flame spheroidisation process. When raw eggshell powder was used as a Ca precursor, the peaks obtained and identified for the microspheres were mainly related to α -TCP as a predominant phase and MgO as a secondary phase in accordance with patterns ICDD 00-009-0348 and 01-071-1176, respectively. XRD peaks of α -TCP, MgO, HA, Mg-WH and Stanfieldite were identified for the samples of E-St(fs)100-160. Moreover, peaks of CaO and some unreacted CaCO_3 were also observed. In addition, predominant HA peaks along with α -TCP as a secondary phase were observed when calcined eggshell powder was used as a Ca precursor. The presence of MgO was also identified in both cases.

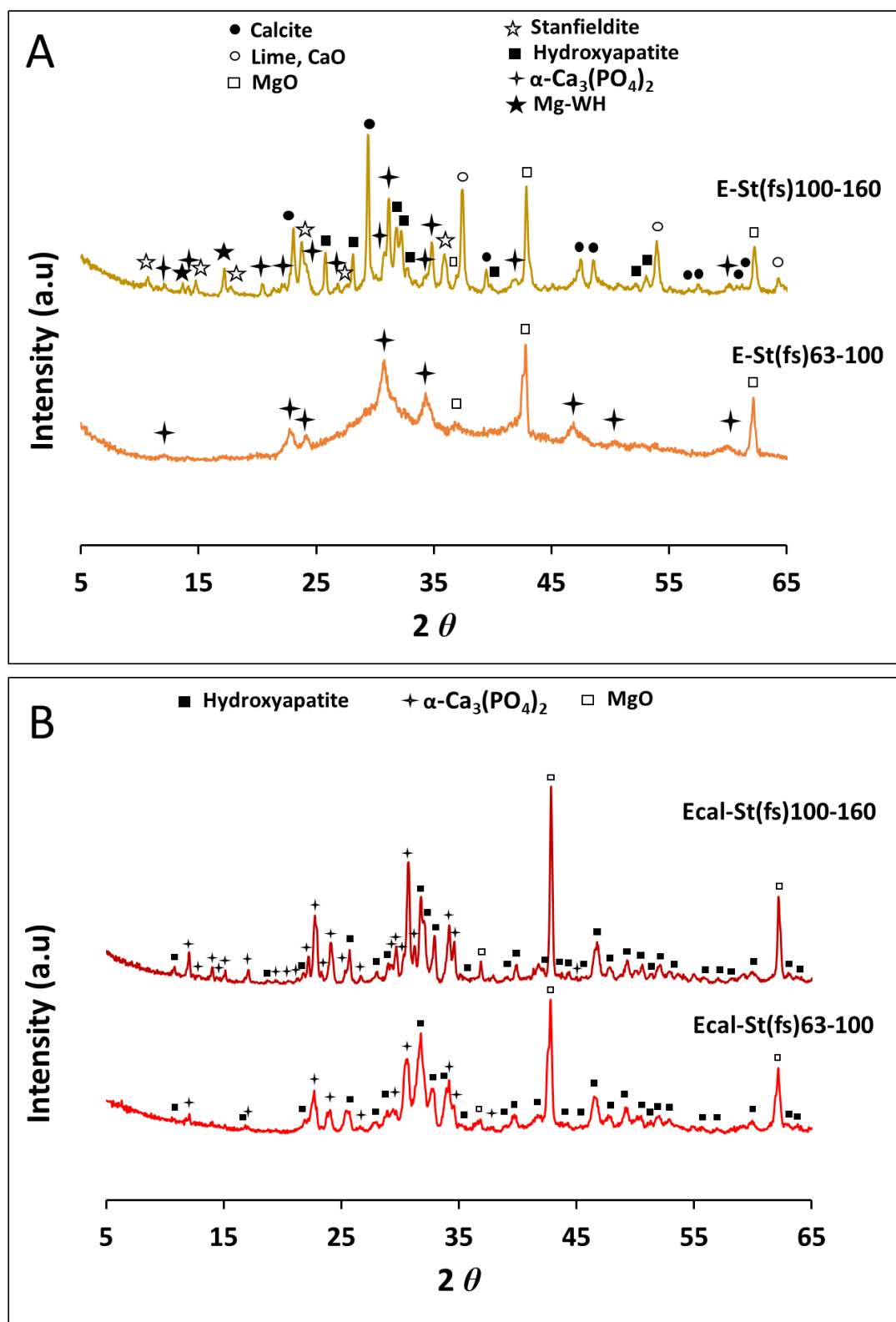


Figure 5.11: XRD pattern of samples synthesised via ball milling followed by flame spheroidisation using (A) raw eggshells and struvite, and (B) calcined eggshells and struvite where two size ranges granules (63 – 100 μm and 100 – 160 μm) of ball milled samples were used for spheroidisation.

5.2.3.4 Functional group analysis by FTIR

The FTIR spectra of the samples obtained via flame spheroidisation are shown in Figure 5.12. The characteristics PO_4^{3-} bands were observed at 565 and 1022 cm^{-1} for all the samples produced [240, 241, 248, 249]. Low resolution bands at 984 cm^{-1} for the anti-symmetric P-O stretching bond and at 954 cm^{-1} for the symmetric P-O stretching bond and a doublet at 560–590 cm^{-1} for the antisymmetric P-O bending bond were also observed [11]. A low resolution peak of CO_3^{2-} was observed at around 1411-1415 cm^{-1} along with band at 875 cm^{-1} for the sample E-St(fs)100-160 [95, 239, 242, 243, 245]. On the other hand, a reduction in the CO_3^{2-} peak at around 1411 cm^{-1} observed for the samples E-St(fs)63-100 and Ecal-St(fs)63-100 [95, 239, 242, 243, 245], whereas this peak almost disappeared for the sample Ecal-St(fs)100-160. In addition, the peak at 875 cm^{-1} was not observed for rest of the samples. The peak positions of the samples and their corresponding assignments are listed in Table 5.6 below.

Table 5.6: FTIR band positions and their corresponding assignments of samples produced via flame spheroidisation process using raw/ calcined eggshells and struvite powder.

Samples and peak positions					References
E-St(fs) (63-100 μ m)	E-St(fs) (100-160 μ m)	Ecal-St(fs) (63-100 μ m)	Ecal-St(fs) (100-160 μ m)	Corresponding assignments	
567, 597	565, 598	565, 569, 596	565, 599	PO ₄ ³⁻ bending (ν_4)	[240, 241, 248, 249]
875	875	875	-	HPO ₄ ⁻ group/ CO ₃ ²⁻ bending (ν_3)	[95, 239, 242, 243, 245, 248, 249, 252]
956	983	956	958, 987	PO ₄ ³⁻ bending (ν_1)	[95, 243, 248-250]
			954	symmetric P--O stretching bond (ν_1) of α -TCP	[274]
			984, 1013	Antisymmetric P-- O stretching bond (ν_3) α -TCP	[274]
987-1050	1022	1022	1022	PO ₄ ³⁻ bending (ν_3)	[95, 243, 250]
1411	1413	1411 1535	1454	CO ₃ ²⁻ bending (ν_3)	[95, 239, 242, 243, 245]
-	3642	-	-	Structural OH ⁻	[249]

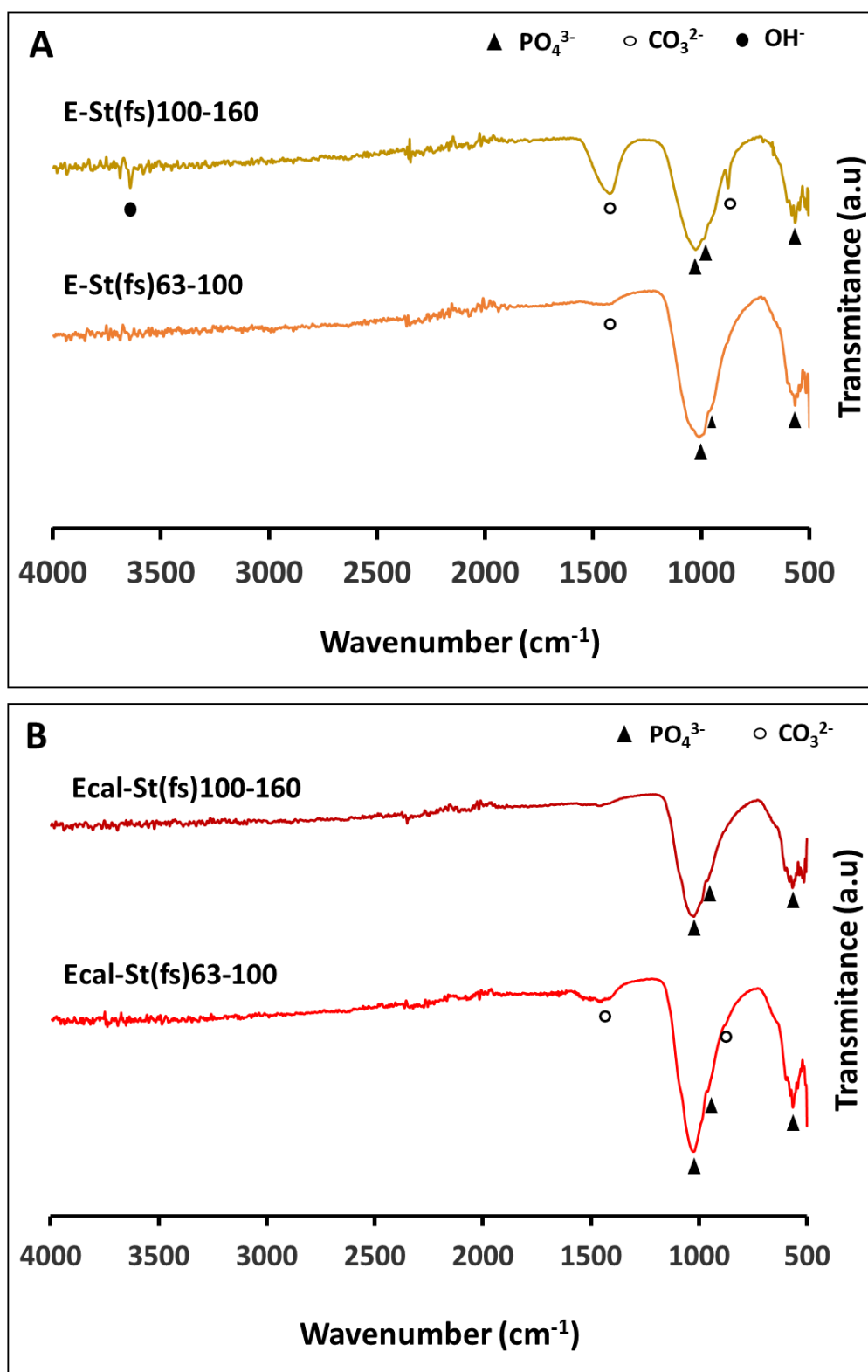


Figure 5.12: FTIR spectra of samples synthesised via ballmilling followed by flame spheroidisation process using (A) raw eggshells and struvite, and (B) calcined eggshells and struvite of two size ranges granules: 63 – 100 μm and 100 – 160 μm .

5.3 Discussion

5.3.1 Overview

In this study eggshells were used to synthesise HA and/or other calcium phosphates considering the advantages and disadvantages of naturally available raw materials as mentioned in the literature review (Chapter 2). Studies in the literature report that HA can be prepared from eggshells using various techniques such as wet chemical methods [243, 275], ball milling [95, 195, 276], hydrothermal [277, 278], microwave [279] and sol-gel [22, 280]. Initial trials for HA preparation from eggshells were explored utilising continuous hydrothermal synthesis processes in chapter 4. After confirmation of formation of nano to submicron HA and Mg-WH with different morphologies (i.e. tube, rod, sheet, elliptical, etc.) from eggshells and struvite (another waste resource) via hydrothermal process, the ball milling method (as a very simple tool) was conducted to synthesise HA and/or other calcium phosphates (CaPs) utilising waste-derived resources of eggshells as Ca and struvite as P sources in this Chapter.

5.3.2 Characterisation of Eggshells

CaCO_3 is one of the main precursors required for the synthesis of CaPs. Eggshells are mainly composed of CaCO_3 . The percentage of CaCO_3 in eggshells was determined via ICP-MS and thermogravimetric analysis (TGA). The compositional analysis via ICP-MS (Table 4.1, Chapter 4) revealed that the CaCO_3 percentage in eggshell powder was around 98 wt% which was consistent with the results obtained via TGA of raw eggshell powder (Figure 5.5 in this chapter). Moreover, the presence of some trace elements, such as Na, Mg and P were also confirmed via ICP-MS (Table 4.1, Chapter 4). These elements are beneficial in biomedical application [85, 151].

The XRD pattern of the eggshell powder (Figure 5.2) correlated well to the calcium carbonate or calcite (CaCO_3) of the rhombohedral form (phase identified from the database was ICDD No. 00-017-0763). The eggshell powder was first calcined at 700 °C and then 900 °C for complete removal of moisture and organic matters. From XRD analysis no significant change between raw eggshell powder and eggshell powder sintered at 700 °C was observed, which

suggested that the CaCO_3 phase was stable up to 700 °C. However, the eggshell powder sintered at 900 °C showed the face-centered cubic form of calcium oxide (CaO) (ICDD No. 00-004-0777) which revealed the decomposition of almost all of the calcium carbonate [281]. The XRD results of eggshell powder also correlated to the FTIR results (Figure 5.4) where the main peak for carbonate and two other peaks for out-plane and in-plane deformation modes due to the presence of calcium carbonate were observed at 1406, 873 and 711 cm^{-1} , respectively [282, 283]. These results are also supported by Tsai *et al.* [282] who studied the chemical and physical properties of eggshells and eggshell membrane particles obtained from hen eggshell waste. They reported that the most significant peak for eggshell particle at 1417 cm^{-1} , was strongly associated with the presence of carbonate minerals within the eggshell matrix. They also reported that the other two peaks observed at 712 cm^{-1} and 875 cm^{-1} , were associated with the in-plane deformation and out-plane deformation modes of calcium carbonate, respectively [282].

Thermogravimetric analysis of raw eggshell powder (see Figure 5.5), revealed a short decreasing plateau of around 1 % weight loss between 40 and 200 °C, which was attributed to moisture evaporation and possibly some organic eggshell membrane decomposition [283]. The second region revealed a slightly steeper slope and a weight loss of 2.2 % was observed from 200 to 600 °C which was suggested to be due to the removal of any remaining organic matter [283]. Furthermore, a sharp decrease in weight loss of around 42.8 % occurred between 600 and 780 °C. This was suggested to be due to the decomposition of calcite (CaCO_3) into calcium oxide (CaO) and carbon dioxide (CO_2) [283]. Thus, total 46% weight loss was observed from room temperature to around 780 °C, after which the percentage of weight loss remained constant up to 1200 °C. This result showed that the entire sample had converted to CaO indicating complete thermal decomposition. In addition, the total percentage of weight loss for eggshell powder sintered at 900 °C was around 4% due to the evaporation of water and potential conversion of unreacted CaCO_3 to CaO and CO_2 . The presence of unreacted CaCO_3 in sintered eggshell powder was confirmed via XRD analysis (see Figure 5.2). Moreover, OH^- groups, observed via FTIR analysis (see Figure 5.4) confirmed the presence of water which had most likely been absorbed by highly reactive CaO to form $\text{Ca}(\text{OH})_2$. Witton [268] also observed OH^- group in FTIR spectrum for eggshells sintered at 900

°C and reported that the presence of OH⁻ group was due to the formation of Ca(OH)₂ during adsorption of water by CaO.

5.3.3 Ball milling followed by sintering

Ball milling of struvite with raw /calcined eggshell powder was performed using deionised water as a milling media. The advantages of wet mechanochemical synthesis are acceleration of dissolution, diffusion, adsorption, reaction rate, and crystallisation by generating high pressure and temperature of local milling zone [284].

The characteristic precursor peaks were only observed in XRD patterns (Figure 5.6A) for the ball milled samples of raw eggshell powder and struvite which revealed that no chemical reaction occurred during ball milling for 2 hr. However, when the samples were sintered at 700 °C, the HA and Farringtonite (Mg₃(PO₄)₂) phases with small amounts of Mg-WH and Stanfieldite formed (Figure Figure 5.6A). The formation of large amount of Farringtonite was due to the thermal decomposition of struvite which occurred by the removal of structural water [H₂O(s)] and structural ammonium [NH₄⁺(s)] [285]. As the decomposition temperature of struvite and calcite is 235 °C and 700-800 °C, respectively [286], the complete removal of structural water and NH₄⁺ occurred at 700 °C with the formation of Mg₃(PO₄)₂ and also some Mg²⁺ and PO₄³⁻ from struvite had reacted with Ca²⁺ of calcite to form calcium phosphates. However, calcite started to decompose slowly at 700 °C and rapidly decompose above 750 °C [287], therefore, a small amount of Ca(OH)₂ was found to be formed while a significant amount of unreacted CaCO₃ was also remained at 700 °C (Figure 5.6A). With a further increase of sintering temperature to 900 °C, almost all the calcite decomposed to CaO, while trace of unreacted calcite was still present (Figure 5.6B). The diffraction peaks indicated increased appearance of Stanfieldite together with small amounts of HA and Mg-WH at 900 °C. However, a complete removal of residual calcite and Stanfieldite was observed when the samples sintered at 1150 °C, which revealed that the Stanfieldite might react with residual CaO (decomposed from remaining calcite) and formed a mixture of HA and Mg-WH at higher temperature of 1150 °C.

One the other hand, the formation of HA with small amount of brucite (Mg(OH)₂) from calcined eggshells and struvite was confirmed via XRD and FTIR (Figure 5.6B and Figure 5.8B).

The characterisation peaks of precursors were not observed confirming that a complete reaction had occurred via ball milling for 3 hr at 400 rpm using calcined eggshells instead of raw eggshells. When this material was sintered at 700 °C, brucite transformed to MgO which was due to the removal of H₂O from Mg(OH)₂ structure [288]. With further increase in sintering temperature to 900 °C, the formation of highly crystalline HA and Mg-WH along with MgO was confirmed via XRD, FTIR, EDX and percentage of crystallinity (Figure 5.6B, Figure 5.8B and Table 5.1, Table 5.2, respectively) as the crystallinity increased with increasing temperature [271]. However, the crystallinity or crystal size of HA remained unchanged when the samples sintered at 1150 °C (Table 5.2) which also correlated with XRD and FTIR by the observation of very similar spectra at both temperatures (i.e. 900 and 1150 °C). The formation of Mg-WH was due to the presence of Mg in struvite. Therefore, the sintering temperature of 900 °C was sufficient to obtain highly crystalline HA with Mg-WH from the calcined eggshells and struvite.

The observation of precursor peaks for the ball-milled raw eggshell-struvite (E-St(bm)0) and the formation of HA for ball-milled calcined eggshell-struvite (Ecal-St(bm)0) might be due to the solubility difference of raw and calcined eggshell powder in water. Raw eggshell powder contained calcium carbonate which is poorly soluble in water (0.013 g L⁻¹ at 25 °C), whereas calcined eggshell powder consisted of CaO and/or Ca(OH)₂ is more soluble (1.59 g L⁻¹ at 25 °C) [289]. As a consequence, the initial reaction rate was slower and less efficient for E-St mixture compared to Ecal-St mixture to obtain desired product (eg, CaPs) [284].

Morphology of the samples prepared from raw eggshells and struvite by ball milling (formed large agglomeration of micron size particles) was clearly distinguished from sintered samples (formed nano to submicron particles, Figure 5.7)) as precursors were decomposed at higher temperature (i.e. struvite at 235 °C and calcite at 700-800 °C) and reacted to each other forming nano products (i.e. HA, Mg-WH and stanfieldite) at 900 °C. However, the particle size increased from nano size (for samples sintered at 900 °C) to sub-micron (for samples sintered at 1150 °C) as particle size increased with increasing temperature as mention above [271].

In addition, nano particles of HA could be possible to produce by simple ball milling of calcined eggshells and struvite [195, 290]. However, particle size increased with increasing sintering temperature due to the increase of crystallinity and bigger particles were also formed at 1150

°C due to the agglomeration of smaller particles. Hamidi *et al.* [254] reported that the sintering of HA powder promoted the particle agglomeration and formed larger particles due to the increased degree of crystallinity. As seen from the FEG-SEM image and XRD pattern, the size of the crystallized particles increased slowly at 700 °C, whereas they grow rapidly at higher temperature (900 and 1150 °C). This phenomenon was due to the fact that the crystallisation is the predominant mechanism at lower temperature range, while the rapid growth of the crystallised particles takes place with a diffusion-controlled mechanism beside crystallisation of other particles [291]. Ahmed *et al.* [291] reported that the diffusion-controlled coarsening of the powder on heating led to grain growth of the crystallised particles resulted in increasing the crystalline size.

In both cases, as seen from the images, the aggregates contained large particles along with some very small particles which might be of Mg-WH (according to crystal size measured). The larger aggregates did not have any definite shape and generated porous agglomerate structure. Similar morphology was reported by Trinkunaite-Felsen *et al.* [271]. They obtained biphasic HA/Mg-WH by sintering dairy calcium powder (DCP) and observed highly porous agglomerate structure. They also mentioned that such type of microstructure of calcium phosphates is beneficial for bone and dental tissues regenerations and for adsorption of hazardous materials from wastewater and nuclear waste disposal [271].

The FTIR spectra of ball milled raw eggshells-struvite samples (Figure 5.7A), exhibited the characterisation peaks of precursor materials only, which was the good agreement with XRD results (Figure 5.6A) of the same sample. The chemical reaction had taken place in between the precursor materials when the ball milled samples were sintered at 700 °C. The peak intensity assigned to Ca-O bond and CO_3^{2-} groups of CaCO_3 decreased significantly, in addition, N-H vibration were completely disappeared as seen at 700 °C compared to pre-sintered samples. However, noticeable peaks related to PO_4^{3-} vibrations had become more pronounced at 700 °C, while another characteristic peak of HA corresponds to OH^- appeared at 628 cm^{-1} confirmed the formation of HA including some other calcium phosphates. With the increase in sintering temperature of 1150 °C, the observation of bands related to PO_4^{3-} , OH^- and HPO_4^{2-} validated the presence of HA and Mg-WH.

FTIR spectra of ball milled samples obtained from calcined eggshells and struvite confirmed the formation of HA and brucite by showing corresponding peaks to different modes of PO_4^{3-} [95, 243, 250], along with a weak peaks and bands of absorbed water [249], respectively. Bands of CO_3^{2-} ion indicated the formation of non-stoichiometric carbonated HA after substituting the PO_4^{3-} ion [95, 239, 242, 243, 245]. For sintered samples (at 700 - 1150 °C), the removal of absorbed water in all sintered samples indicated the conversion of brucite to MgO [292]. Sharp peaks of PO_4^{3-} and OH^- confirmed the formation of highly crystalline HA for all the sintered samples as also confirmed via XRD pattern. However, the high intensity peak at 1088 cm^{-1} for the samples obtained at 900 and 1150 °C might be due to the presence of HPO_4^{2-} (non-stoichiometric) group in Mg-WH [263]. The CO_3^{2-} band at around 1400 and 875 cm^{-1} were found to be decreased at 700 °C, while these bands completely disappeared after sintering at higher temperature of 900 and 1150 °C. This can be attributed that the CO_3^{2-} ion fully decomposed at higher temperature to CO_2 [283] and formed only reaction products of HA, Mg-WH and MgO.

The thermal behaviour of the two samples produced via ball milling of struvite with raw and calcined eggshell powder (<45 μm), respectively had been compared via TGA/DSC. The occurrence of major wt loss of 25 % up to 200 °C for the ball milled samples of raw eggshells and struvite can be attributed to the decomposition of struvite (start at around 50 °C) [29]. This mass loss corresponded to the following decomposition reaction for struvite (Eq. (1)) [293]:



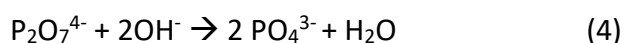
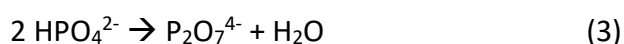
However, the moisture evaporation from calcite (coming from raw eggshell powder) and possibly some organic eggshell membrane decomposition occurred in this temperature range [283]. The second region in between 200 and 400 °C had a short decreasing plateau with the wt loss of around 5 % which was suggested to be due to the removal of any remaining organic matter [283]. Furthermore, a sharp decrease in wt loss of around 17 % from 600 to 744 °C was due to the starting of decomposition of calcite (CaCO_3) [283].

An approximate 47 wt% loss was observed for the E-St(bm) from room temperature to around 744 °C, after which the percentage weight loss remained constant up to 1200 °C. This result suggested that the thermal decomposition of the samples had been completed. However,

some residual peaks of CaCO_3 and CaO observed in XRD pattern for the ball milled samples (raw eggshells and struvite) when sintered at 900°C .

On the other hand, the ball milled samples prepared using calcined eggshells and struvite consists of hydroxyapatite and $\text{Mg}(\text{OH})_2$. In the temperature range from room temperature to 200°C , a 10 wt% loss was attributed to the desorption of physically adsorbed water and the vaporisation of the residual organic compounds [294]. The following weight loss stage, from 200 to 400°C , corresponding to 5.5 wt% loss, was likely due to the loss of adsorbed and lattice water and decomposition of $\text{Mg}(\text{OH})_2$ to MgO and H_2O [292]. Further 6.1 wt% loss from 400 to 960°C was also attributed to the decomposition of remaining $\text{Mg}(\text{OH})_2$ [292].

In addition, the mass loss at this region also correlated to the removal of interstitial water due to disintegration of the HPO_4^{2-} group present in the non-stoichiometric HA and Mg-WH, according to the following reactions [294]:



However, there was only 0.4 wt% loss on heating up to 1200°C indicated the high thermal stability of the sample. This results also correlated to XRD and FTIR data where, no significant difference in XRD and FTIR spectra for the sintered ball milled (calcined eggshells and struvite) samples at 900 and 1150°C was observed.

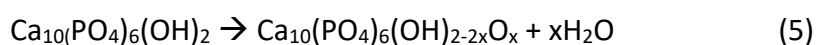
5.3.4 Flame spheroidisation

Interestingly, ball milled samples were fully transformed into microspheres (Figure 5.9) via flame spheroidisation process, which was due to the surface tension forces during the cooling stage as the molten particles were ejected from the flame [31]. Higher yield of microspheres was observed for the smaller starting granules (i.e. $63\text{-}100\ \mu\text{m}$) compared to the larger granules ($100\text{-}160\ \mu\text{m}$) due to the lower heat energy required to transform into microspheres for smaller particles compared to larger particles [295]. The α -TCP along with MgO phase containing microspheres formed for the starting granules of smaller size range ($60\text{-}100\ \mu\text{m}$), when raw eggshell powder was milled with struvite. Whereas four types of CaPs such as HA,

α -TCP, Stanfieldite and Mg-WH formed for the larger granules (100-160 μm) (as confirmed via XRD and FTIR, see Figure 5.11 and Figure 5.12). As the flame spheroidisation process involves high temperature ($\sim 3100^\circ\text{C}$), the process helped to decompose the eggshells to CaO which reacted with struvite and formed different types of CaPs (HA, α -TCP) including Mg containing CaPs. The formation of Stanfieldite and Mg-WH was due to the presence of Mg in the P precursor. In addition, the observation of calcite and CaO peaks indicated the presence of some unreacted CaCO_3 and CaO, which could be due to ball milled samples that had not passed through the flame (i.e. had scattered from the surface of the flame due to high gas pressure).

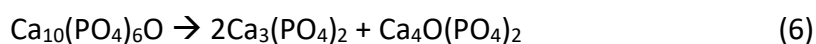
On the other hand, the Ca precursor completely converted to HA and α -TCP microspheres via flame spheroidisation when the calcined eggshell powder was milled with struvite (Figure 5.11B). As ball milled calcined eggshell-struvite powder contained HA and brucite ($\text{Mg}(\text{OH})_2$) (Figure 5.6B), it is suggested that some HA converted to α -TCP and brucite to MgO due to the high temperature and rapid processing of the flame spheroidisation process. The surface phosphorus of HA was reactive and readily formed P-OH group which were dehydroxylated when heated to form surface P-O-P group [296].

Bulina *et. al.* [297] reported that the process of HA dehydroxylation with the release of water started at $850\text{--}900^\circ\text{C}$ and led to the formation of vacancies in the position of the hydroxyl groups in the HA structure. As a result of the formation of oxyhydroxyapatite (OHA) had taken place by the following reaction:



When the apatite structure has no hydroxyl groups, i.e, $x = 1$ in equation (1), an oxyapatite (OA) $\text{Ca}_{10}(\text{PO}_4)_6\text{O}$ is formed.

At the temperature 1300°C led to decomposition of the OHA with the formation of α -TCP and tetracalcium phosphates, and calcium oxide by the following reactions [298]:



Therefore, it is suggested that HA was partially converted to α -TCP at high temperature during the flame spheroidisation and rapid cooling process. The formation of Mg containing CaPs (in E-St(fs)100-160) was due to the presence of Mg in struvite as mentioned above and which was also converted to MgO at high temperature.

The formation of α -TCP was also confirmed via EDX analysis (Figure 5.10, Table 5.4) where the Ca/P ratio was 1.5 for E-St(fs)63-100, which is a characteristic value of pure TCP and ≥ 1.6 for rest of the samples as HA and/or other calcium phosphates formed which were also in good agreement with XRD (Figure 5.11). The higher Ca/P ratio of 1.65 for the samples (E-St(fs)100-160) produced from larger particles indicated that excess Ca was due to the presence of residue of the Ca precursor [298] as shown via XRD patterns (Figure 5.11). For the same reason unreacted CaCO_3 particles as a residue from the precursor materials, high resolution of CO_3^{2-} peak at 1411 and 875 cm^{-1} in sample E-St(fs)100-160 was observed via FTIR (Figure 5.12).

The distortion of the doublet peak at around 550-605 cm^{-1} observed in E-St(fs)63-100 was attributed to a lower order of crystallinity in TCP [298]. However, comparatively a sharp peak at around 1022 cm^{-1} (characteristics bands of the PO_4^-) indicated the formation of higher percentages of HA for Ecal-St(fs)63-100 as compared to the other three samples produced via flame spheroidisation.

In summary, nano to submicron HA and Mg-WH particles and/or microspheres were prepared via simple ball milling of waste-derived resources of eggshells (as Ca source) and struvite (as P source) followed by sintering or via the rapid (solvent-free) flame spheroidisation process. Moreover, HA could be prepared via simple ball milling of calcined eggshells and struvite. These materials and methods serve as a cheap and effective ways to produce biomaterials which could potentially be used for biomedical applications [299].

Chapter 6

Fabrication of Hydroxyapatite (HA)/ Calcium Phosphates (CaPs) - Chitosan (Ch) composites

6.1 Introduction

After the successful production of various morphologies of CaPs using wastes such as eggshells and struvite via three different methods (hydrothermal synthesis, ball milling followed by sintering, and ball milling followed by flame spheroidisation process), fabrication of composites using commercial chitosan (Ch) was explored to produce scaffolds and films in Chapter 6. The CaPs used were selected from the products obtained via each processing method mentioned above and commercial hydroxyapatite (HA) was also used as a control. Moreover, the scaffolds fabricated (except for hydrothermally synthesised CaP) were crosslinked using naturally derived crosslinking agent (genipin) to improve their mechanical properties and stability in an aqueous environment. This Chapter includes two experimental sections: the first section describes preparation process of the scaffolds, whilst the second section describes the composite films preparation, as highlighted in the flowchart below (see Figure 6.1).

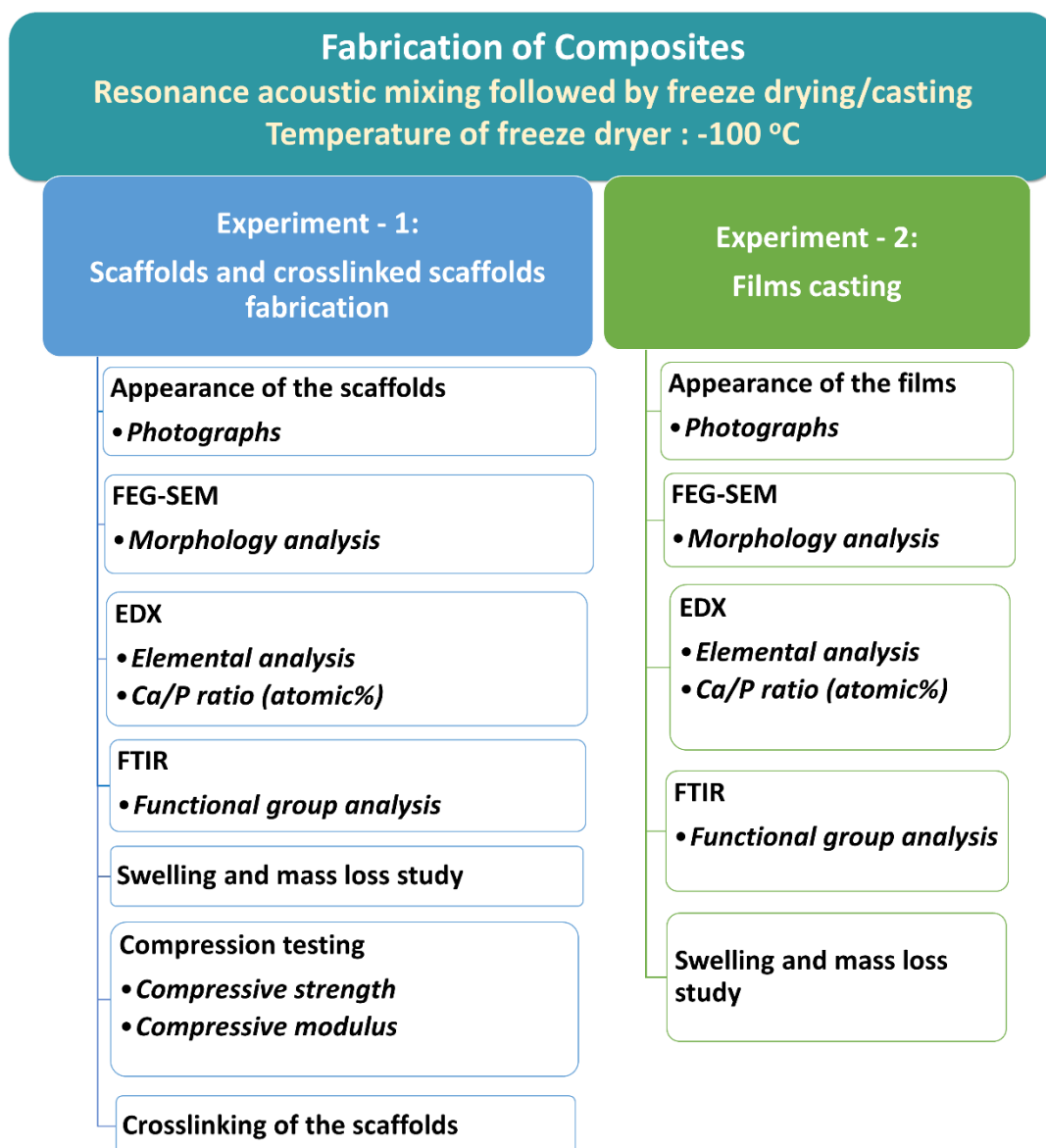


Figure 6.1: Schematic flow chart of experimental sections including characterisation techniques used for the scaffolds and films fabricated from HA/CaPs and chitosan via resonance acoustic mixing followed by freeze drying and solvent casting, respectively.

6.2 Results

The sample codes of the composites fabricated, and the HA/CaPs produced including their preparation methods and phases obtained are highlighted in Table 6.1.

Table 6.1: Sample codes of the composites fabricated, and the HA/CaPs selected including their preparation methods and phases obtained.

Composite Sample codes			HA/CaPs samples used			
Scaffolds	Crosslinked scaffolds	Films	Methods	Selected samples	Phases	Ca/P
HA _{com} -Ch	HA _{com} -Ch/G	(F)HA _{com} -Ch	Hydrothermal synthesis (Commercial)	HA _{com} (Promethean particles Ltd.)	Nano hydroxyapatite (nHA)	1.6
CaP _{ht} -Ch			Hydrothermal synthesis	CaP _{ht} : E/St(Ac-N)350	Mg-WH, HA	1.72
CaP _{bm} -Ch	CaP _{bm} -Ch/G	(F)CaP _{bm} -Ch	Ball milling followed by sintering	CaP _{bm} : Ecal-St(bm) ($<25\mu\text{m}$)	HA, Brucite (Mg(OH) ₂)	1.52
CaP _{fs} -Ch	CaP _{fs} -Ch/G	(F)CaP _{fs} -Ch	Flame spheroidisation	CaP _{fs} : Ecal-St 63-100	MgO, α -TCP, HA	1.58

6.2.1 Scaffold Production

Three different sets of scaffolds were prepared using chitosan (Ch) and CaPs obtained via hydrothermal synthesis (ht), ball milling followed by sintering (bm) and ball milling followed by flame spheroidisation (fs) (in microsphere form). Another control group of scaffolds were also produced using Ch and commercial HA. The representative images of the scaffolds developed is shown in Figure 6.2.

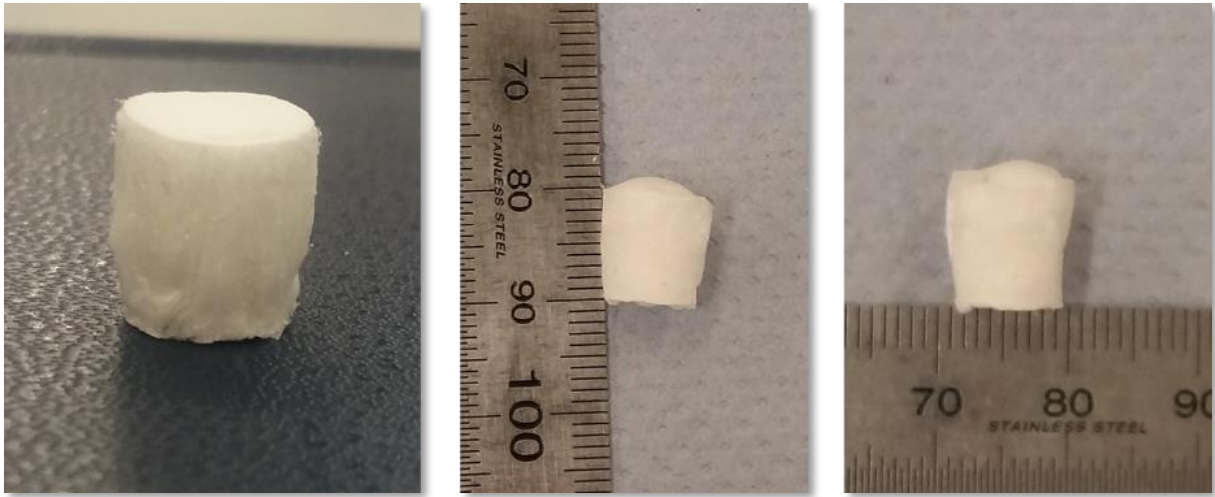


Figure 6.2: The representative optical image of a scaffold fabricated.

6.2.1.1 Morphology characterisation using FEG-SEM

Longitudinal and transverse cross-sections of the scaffolds fabricated were obtained to examine their internal morphology via SEM analysis as shown in Figure 6.3 A1 – D1 and Figure 6.3 A2 – D2, respectively. Highly porous microstructures with interconnected porosity were observed for all the scaffolds prepared. The nanocrystalline HA/CaPs were distributed homogeneously (indicated by yellow arrows) through the Ch matrix which was clearly observed in Figure 6.3A3- 3C3. Moreover, the microspheres were clearly seen embedded in the Ch matrix, in Figure 6.3D1 and D2.

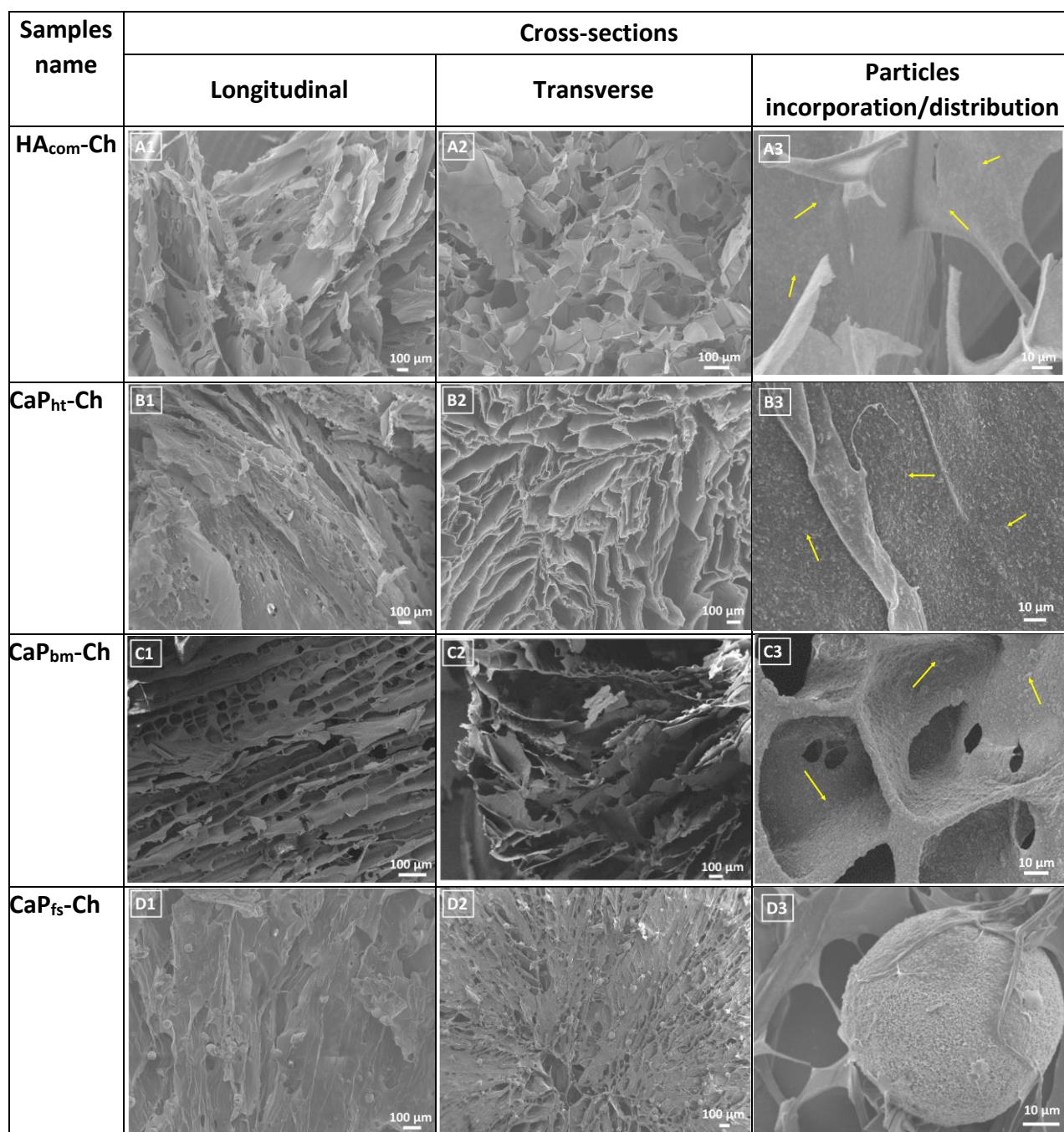


Figure 6.3: Longitudinal (A1-D1) and transverse (A2-D2) cross-sectional morphology of scaffolds fabricated via resonance acoustic mixing followed by freeze drying. Images A3-D3 show the HA/CaPs particles distribution (indicated by yellow arrows) in the chitosan (Ch) matrix at higher magnification (10 μ m).

6.2.1.2 Pore size of scaffolds

Pore sizes of four different scaffolds prepared from Ch and HA/CaPs were measured using ImageJ software and presented in Table 6.2. A range of 20 – 50 pores from different images of each sample were selected to measure length and width of the pores. Pore size ranges for the HA_{com}-Ch (31-169 μm) were found to be higher than the CaP_{bm}-Ch (7-65 μm).

Table 6.2: Pore size of four different scaffolds prepared from Ch and HA/CaPs via resonance acoustic mixing followed by freeze drying.

Samples code	HA _{com} -Ch	CaP _{ht} -Ch	CaP _{bm} -Ch	CaP _{fs} -Ch
Pore size (n=20-50) μm	31-169	16-112	7-65	11-375

6.2.1.3 Elemental analysis via EDX

The EDX spectra and the atomic % of elements from where the Ca/P ratios of the scaffolds fabricated were determined are presented in Figure 6.4 and Table 6.3, respectively. The corresponding EDX spectrum revealed that the chemical elements of the HA/CaP-CH scaffolds were mainly composed of O, Ca, P, Mg (derived from HA/CaP) and N (derived from Ch). The Ca/P atomic (%) ratios for commercial HA and synthesised CaPs in the scaffolds were found to be in the range of 1.51 (for CaP_{fs}-Ch) -1.72 (for CaP_{ht}-Ch).

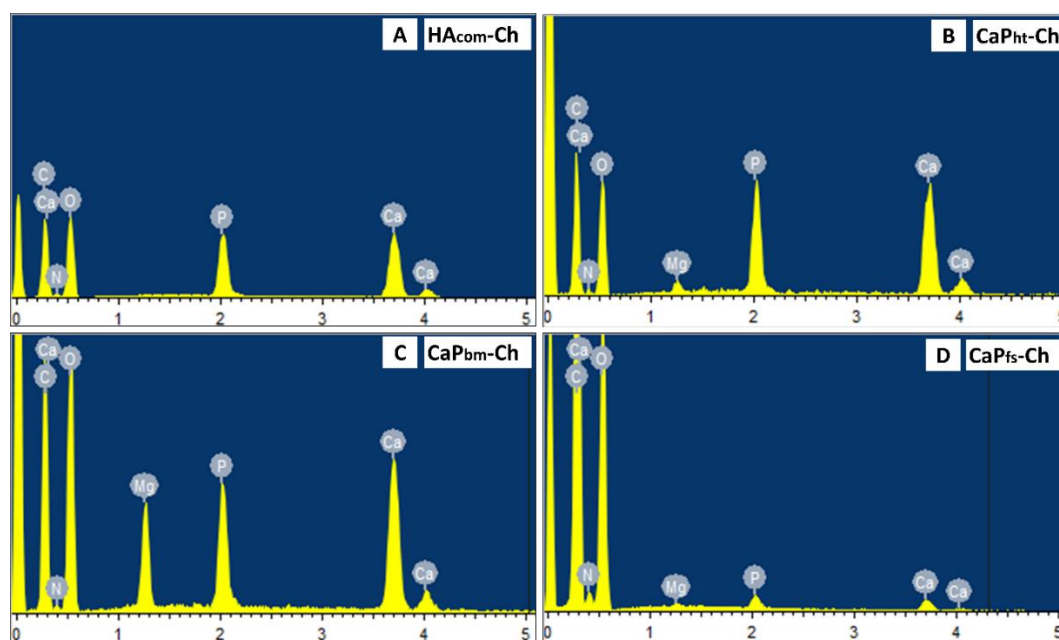


Figure 6.4: EDX spectra of the scaffolds A) HA_{com}-Ch, B) CaP_{ht}-Ch, C) CaP_{bm}-Ch and D) CaP_{fs}-Ch fabricated from HA/CaPs and chitosan via resonance acoustic mixing followed by freeze drying.

Table 6.3: Elemental percentage (in atomic%) and Ca/P ratios of the scaffolds fabricated via resonance acoustic mixing followed by freeze drying.

Samples name	Elements present in the scaffolds (in atomic%)					Obtained Ca/P	Expected based on samples used
	O	Ca	P	Mg	N		
HA _{com} -Ch	61.83	9.05	5.71	0.02	23.05	1.59	1.6
CaP _{ht} -Ch	53.27	11.36	6.61	0.72	28.03	1.72	1.72
CaP _{bm} -Ch	64.32	8.95	5.64	5.71	15.23	1.59	1.52
CaP _{fs} -Ch	75.33	1.29	0.85	0.3	22.13	1.51	1.58

6.2.1.4 Functional group analysis by FTIR

To study the functional groups present amongst the components present in the scaffolds, Fourier transform infrared spectroscopy (FTIR) was carried out as presented in Figure 6.5 and Table 6.4.

The FTIR spectra of pure chitosan showed a broad band at around 3100-3550 cm^{-1} with the highest peak at 3290 cm^{-1} which corresponded to $-\text{NH}_2$ and $-\text{OH}$ stretching vibrations. Peak at 2869 cm^{-1} assigned to C-H stretch vibration [46, 216]. The bands at 1658 cm^{-1} and 1587 cm^{-1} represented the C=O stretching vibration (amide I) [226, 300] and NH bending vibration of primary amine group (amide II) [46, 216, 226, 300] respectively. Bands found at 1413 and 1375 cm^{-1} indicated the CH_2 bending and CH_3 symmetrical deformations respectively [301], while band at 1315 cm^{-1} indicated C-N vibration (amide III) [301, 302] and 1149 cm^{-1} attributed to asymmetric stretching of the C-O-C bridge [301]. The characteristic peaks at 1061 and 1024 cm^{-1} associated with the C-O vibration [46, 301] were also observed for chitosan. The band at 892 cm^{-1} was attributed to the pyranose ring skeletal vibrations [303].

Commercial hydroxyapatite showed the characteristics peaks of different modes of the PO_4^{3-} group at around 1000–1100 (962, 1018, 1089) cm^{-1} and 500–600 cm^{-1} (561, 599) [304]. A weak peak was observed at 869 cm^{-1} which was assigned to the stretching vibration of CO_3^{2-} group. A small and sharp peak was identified at 3572 cm^{-1} and 630 cm^{-1} , corresponding to the stretching mode of the $-\text{OH}$ group [216].

The peaks for the bending ν_4 , ν_1 and ν_3 vibration of PO_4^{3-} group were identified between 557-611 cm^{-1} , 962-968 cm^{-1} and 1022-1031 cm^{-1} for HA_{com}-Ch and CaP_{ht}-Ch suggesting the

presence of CaPs phases [300, 305], whereas the lower intensity of these peaks was found for the $\text{CaP}_{\text{bm}}\text{-Ch}$ and disappeared for the $\text{CaP}_{\text{fs}}\text{-Ch}$. However, peaks at around $1022\text{-}1031\text{ cm}^{-1}$ also corresponded to the C-O stretching vibration of Ch [305, 306]. The characteristic peaks of Ch was identified for all the scaffolds at around 1377 , 1406 , 1550 , and 1637 cm^{-1} attributed to the $-\text{CH}_3$ bending vibration, the $-\text{CH}_2$ symmetrical deformation mode, amide II and amide I, respectively. All the scaffolds showed a prominent broad band at around 3363 cm^{-1} and a low intense band at around 2866 cm^{-1} , which were ascribed to the O-H and N-H symmetrical vibration and C-H stretching vibrations (symmetric and asymmetric) of Ch [300, 307]. However, all these characteristic bands of Ch were observed in low resolution for the $\text{CaP}_{\text{bm}}\text{-Ch}$ sample.

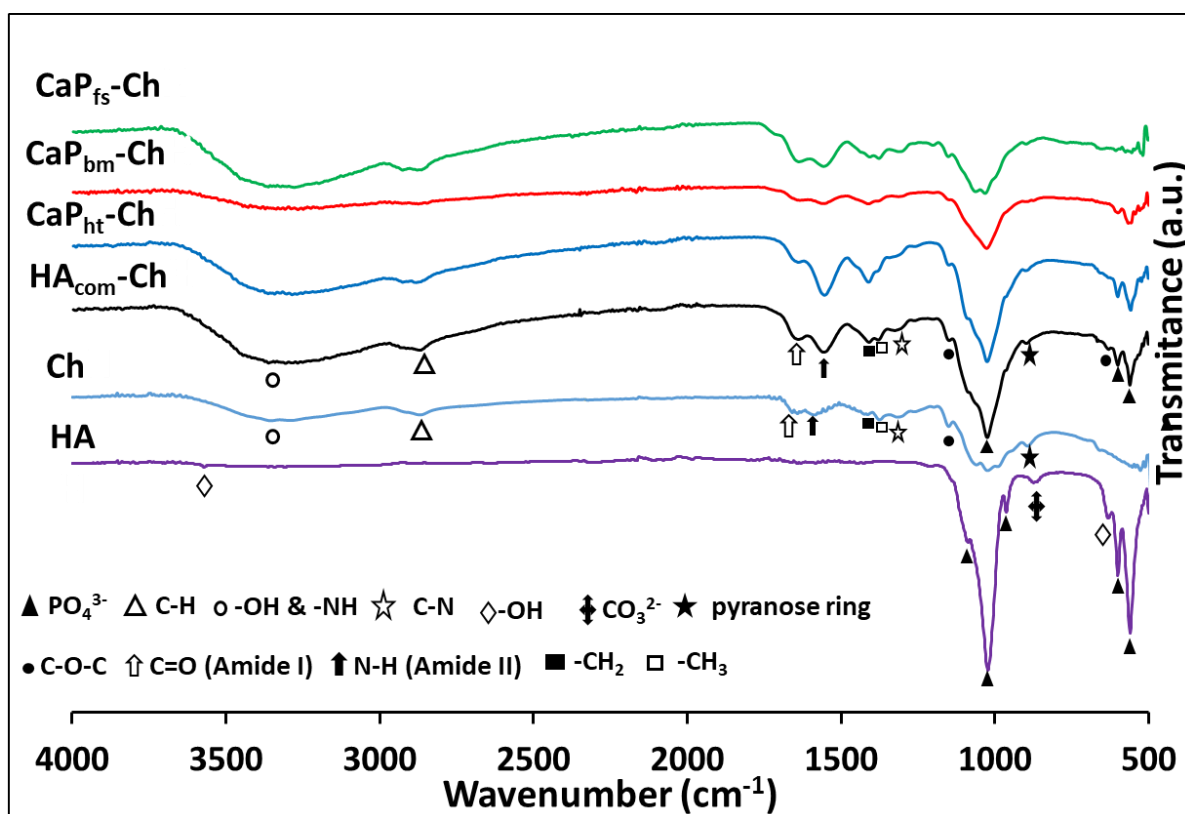


Figure 6.5: FTIR spectra of four different types of scaffolds fabricated via resonance acoustic mixing followed by freeze drying.

Table 6.4: FTIR band positions and their corresponding assignments of scaffolds produced via resonance acoustic mixing followed by freeze drying.

Sample names/peak positions				Corresponding assignments	References
HA _{com} -Ch	CaP _{ht} -Ch	CaP _{bm} -Ch	CaP _{fs} -Ch		
561, 599	557, 600	557, 600	555 - 600	the bending vibration (ν_4) of the phosphate (PO_4^{3-}) groups	[300, 305]
630	631		-	Structural -OH	[305]
897	901	-	898	C-H bending (Out-of-plane) (CH)	[308]
1024	1026	1026	1031, 1062	C-O stretching (CH) / the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups	[305, 306]
1132	1149	1147	1151	C-O-C stretching mode	[305, 306]
1325	1342	1313	1312	C-N stretching (CH)	[308]
1379, 1408	1377, 1410	1383, 1410	1377, 1406	the $-\text{CH}_3$ bending vibration and the $-\text{CH}_2$ symmetrical deformation mode	[300]
1556, 1637	1552, 1637	1554, 1639	1556, 1637	amide II and amide I	[300]
2866	2868	2847	2864, 2926	could be attributed to C-H stretching vibrations (symmetric and asymmetric)	[307]
3200 - 3400	3260 - 3360	3200 - 3400	3260 - 3360	O-H and N-H symmetrical vibration	[300]

6.2.1.5 Swelling and mass loss study of the scaffolds

The swelling properties of the scaffolds was carried out using PBS for up to 96 hours and the results are presented in Figure 6.6. It was observed that the highest water uptake (445 %) at 2 hrs was for the commercial HA-Ch scaffolds (HA_{com}-Ch) which decreased to 390 % at 5 hrs and then remained constant for the remainder of the time. Similar trend was observed for the CaP_{fs}-Ch with highest water uptake of 420 % within first 15 min which then decreased to 186 % till 6 hrs and then remained constant. Water uptake for the CaP_{ht}-Ch was observed to be 426 % at 6 hrs which then revealed an increase to 524 % at 96 hrs. The CaP_{bm}-Ch showed 234 % water uptake at 3 hrs and then remained almost constant for the rest of the time.

The percentage mass losses of the scaffolds measured after 96 hrs immersion in PBS are presented in Figure 6.7. The highest mass loss (35 %) for the CaP_{fs}-Ch and the lowest mass loss (3.84 %) for the CaP_{ht}-Ch was observed.

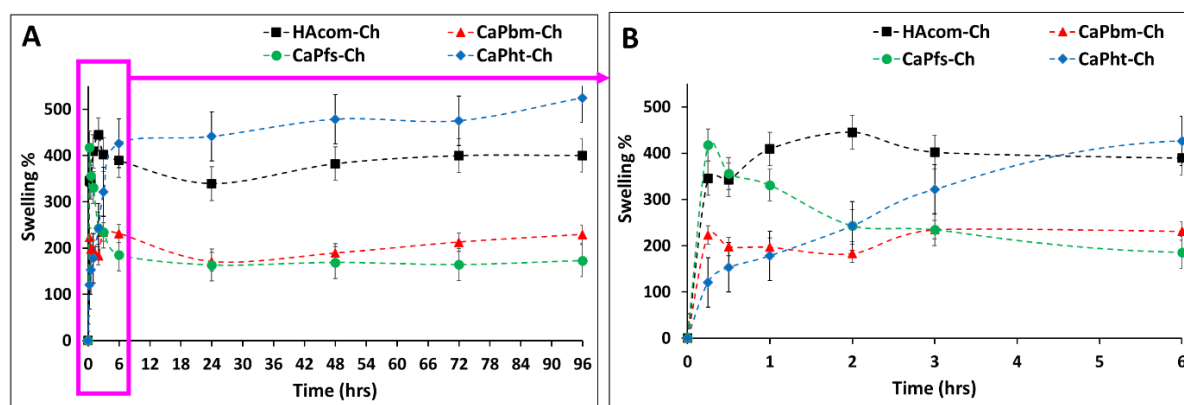


Figure 6.6: Swelling properties of four different scaffolds fabricated measured in PBS upto 96 hrs (A) and first 6 hrs (B).

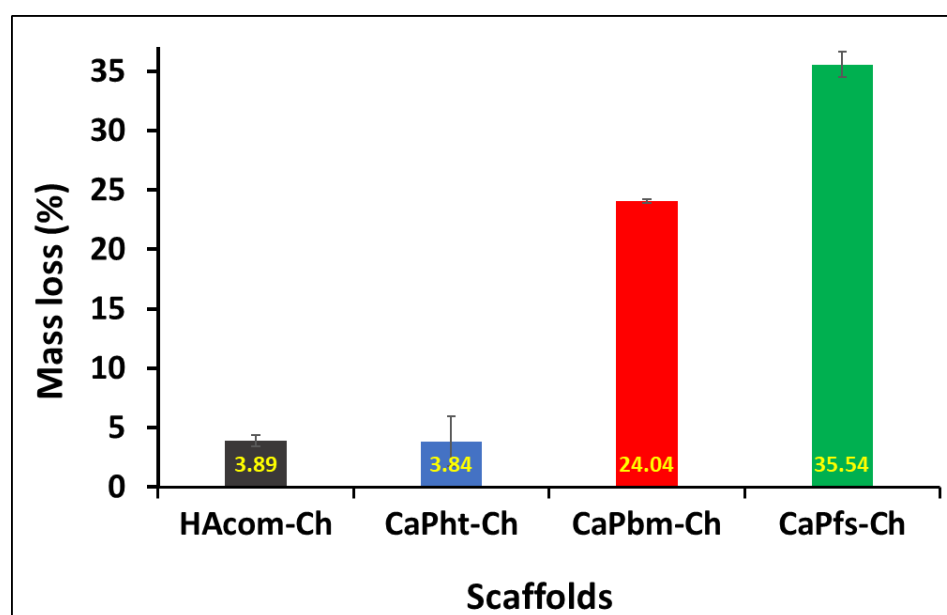


Figure 6.7: Mass loss of the scaffolds fabricated after 96 hrs of immersion in PBS and dried overnight at 50.

6.2.1.6 FTIR analysis after swelling test

FTIR spectra of scaffolds after 96 hrs of immersion in PBS are presented in Figure 6.8. Lower intensities of the absorption bands for all the scaffolds compared to their corresponding scaffolds (before immersion in PBS, Figure 6.5) were observed.

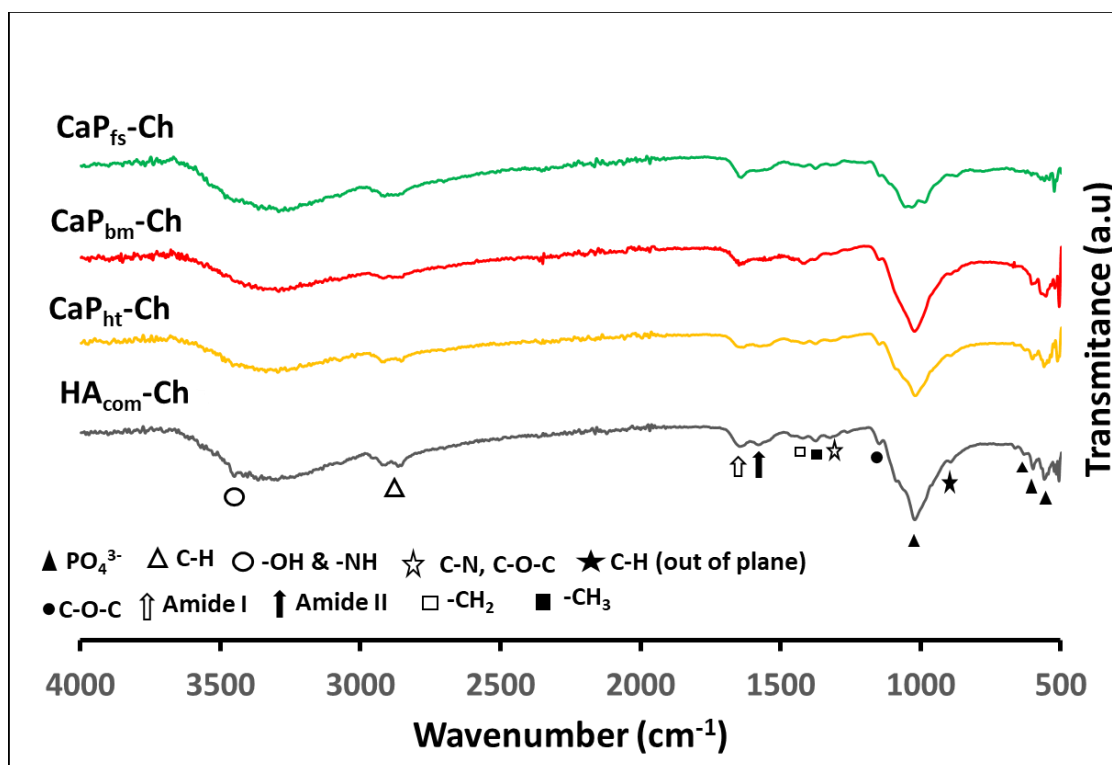


Figure 6.8: FTIR spectra of the scaffolds after swelling test for 96 hrs.

6.2.1.7 Mechanical properties

Compression testing of the dry scaffolds (as shown above in Figure 6.2) was carried out to measure the compressive mechanical properties, which are shown in Figure 6.9. The compressive strength of the scaffolds was found to be within the ranges of 6 (for HA_{com}-Ch) and 26 kPa (for CaP_{bm}-Ch). The highest compressive modulus was obtained for CaP_{bm}-Ch (0.79 kPa) and lowest for the HA_{com}-Ch (0.04 kPa).

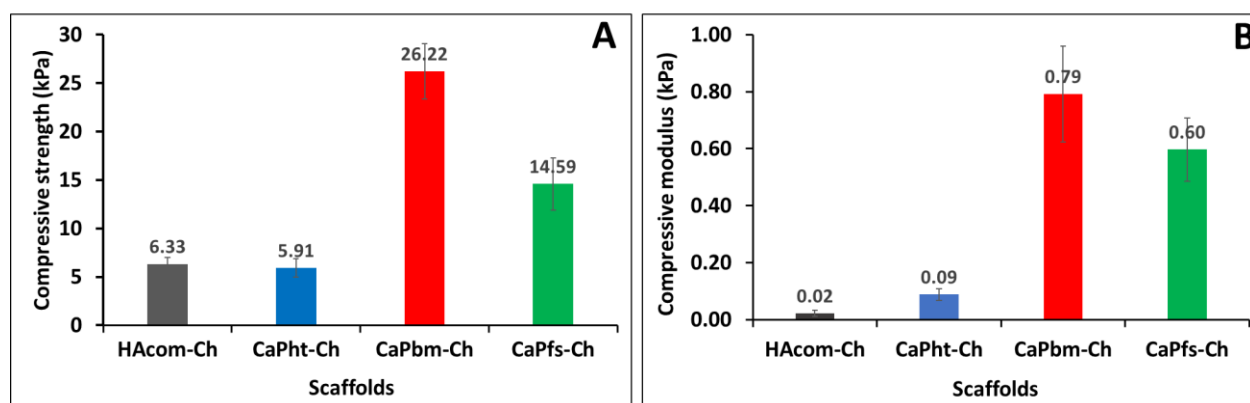


Figure 6.9: Compressive strength (A) and compressive modulus (B) of the scaffolds fabricated from commercial chitosan (Ch) and commercial hydroxyapatite (HA) or calcium phosphates (CaPs) obtained from eggshells and struvite via hydrothermal synthesis (ht), ball milling (bm) and flame spheroidisation (fs).

6.2.2 Crosslinking of Scaffolds

Crosslinking of the scaffolds prepared was carried out to investigate the effect of crosslinking with genipin (G) on the stability and mechanical properties of the scaffolds.

6.2.2.1 Appearance of crosslinked scaffolds

Three scaffold types (HA_{com}-Ch, CaP_{bm}-Ch and CaP_{fs}-Ch) were crosslinked using a naturally derived (fruit of *Gardenia jasminoides*) crosslinking agent genipin (G). Images of the crosslinked scaffolds are shown in Figure 6.10.

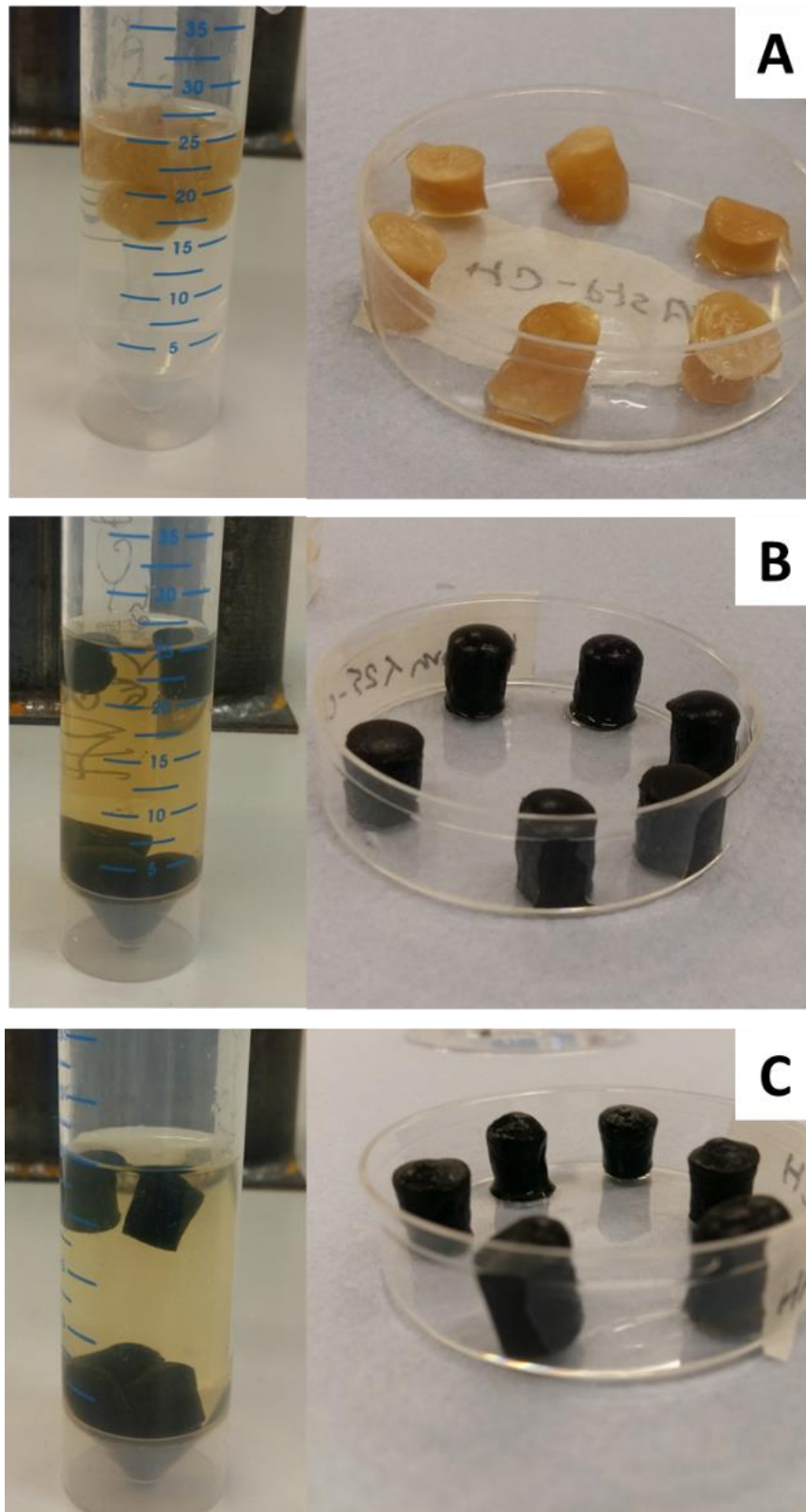


Figure 6.10: Images of crosslinked scaffolds (A) $HA_{com}-Ch/G$, (B) $CaP_{bm}-Ch/G$ and (C) $CaP_{fs}-Ch/G$ after 24 hrs immersion in genipin (G).

6.2.2.2 Morphology characterisation using FEG-SEM

The internal morphologies of the crosslinked scaffolds (longitudinal and transverse sections) are presented in Figure 6.11. SEM micrographs shows that the crosslinked scaffolds have smaller pore sizes compared to their respective non-crosslinked scaffolds.

6.2.2.3 Pore size of cross-linked scaffolds

Pore sizes of the three different cross-linked scaffolds prepared from Ch and HA/CaPs using genipin were measured using ImageJ software and presented in Table 6.5. Pore size ranges were found to be higher for the HA_{com}-Ch/G (6-146 μm) compared to the CaP_{bm}-Ch/G (5-54 μm).

Table 6.5: Pore size of the three different cross-linked scaffolds prepared from Ch and HA/CaPs using genipin via resonance acoustic mixing followed by lyophilisation.

Samples code	HA _{com} -Ch/G	CaP _{bm} -Ch/G	CaP _{fs} -Ch/G
Pore size (n = 20 - 50) μm	6 - 146	5-54	11 - 98

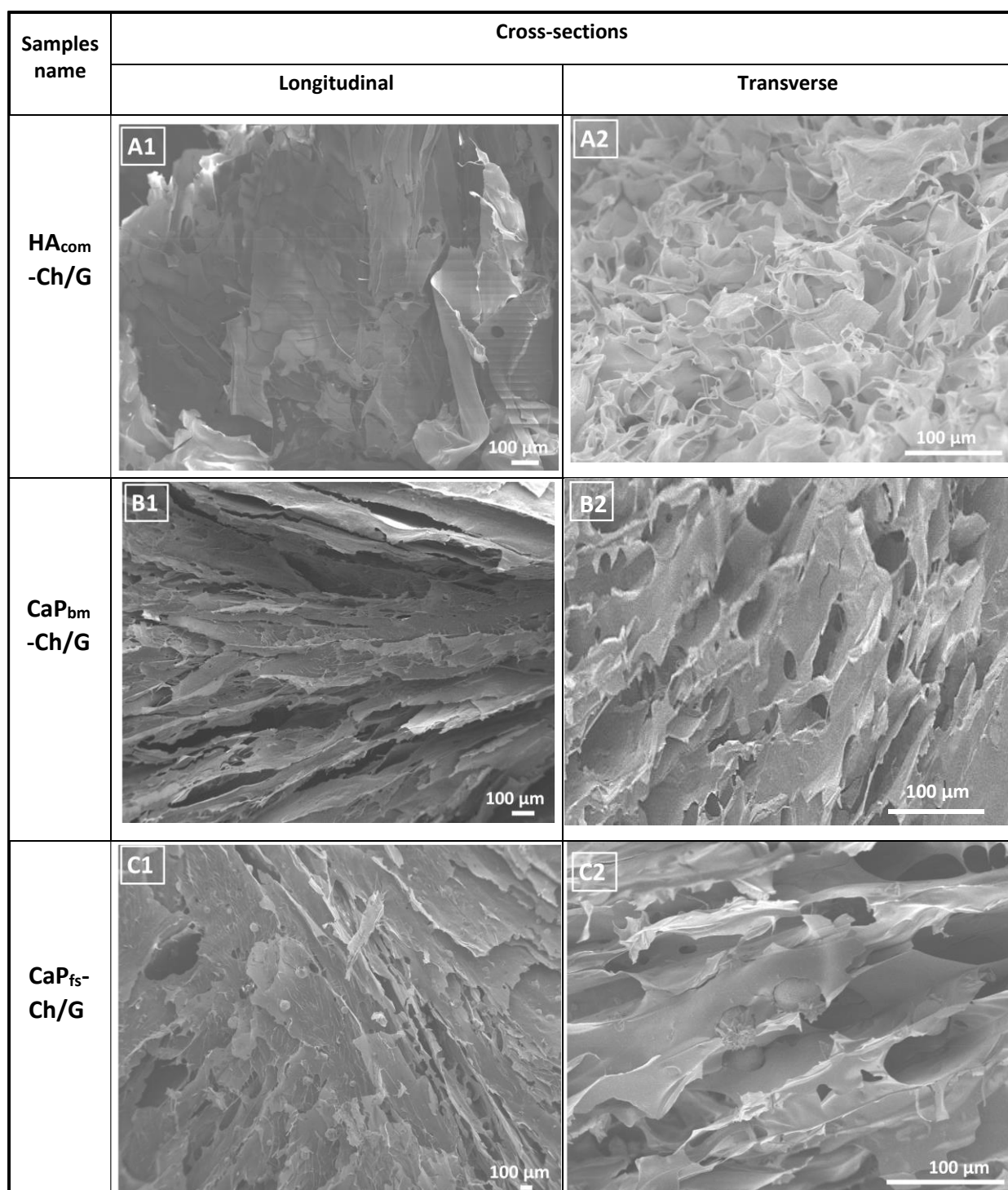


Figure 6.11: Longitudinal (A1-C1) and transverse (A2-C2) cross-sectional morphology of the crosslinked scaffolds fabricated via resonance acoustic mixing followed by lyophilisation.

6.2.2.4 Elemental analysis via EDX

The atomic % of elements from the EDX spectra and the Ca/P ratios of the cross-linked scaffolds fabricated as presented in Figure 6.12 and Table 6.6, respectively. The corresponding EDX spectrum revealed that the chemical elements of the crosslinked HA/CaP-Ch scaffolds were mainly composed of Ca, P, Mg, O (derived from HA/CaP) and N (derived from Ch). The Ca/P atomic ratios for commercial HA and synthesised CaPs in the cross-linked scaffolds were found to be in the range of 1.43 (for CaP_{bm}-Ch/G) -1.55 (for HA_{com}-Ch/G).

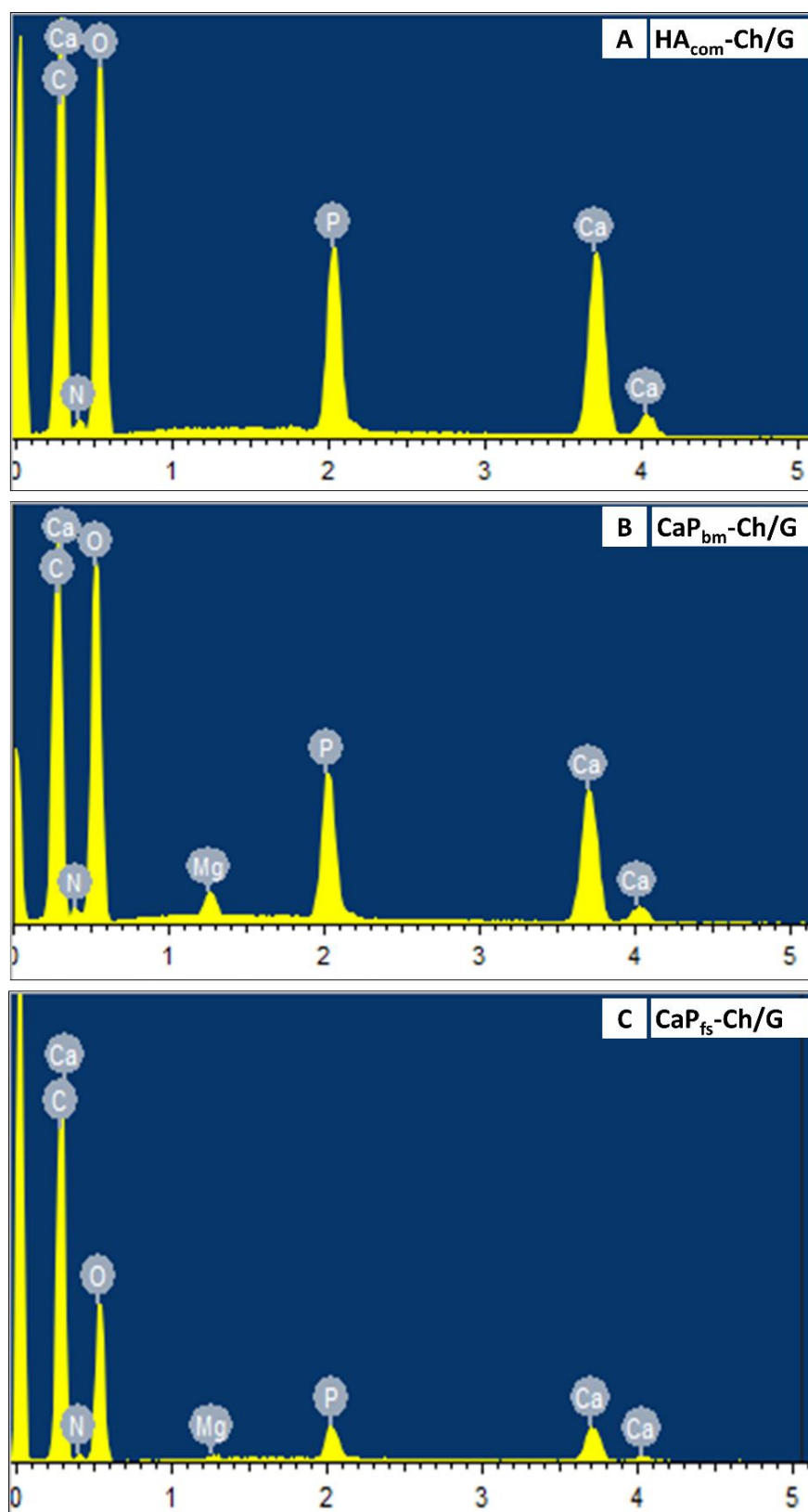


Figure 6.12: EDX spectrum of the cross-linked scaffolds (A) HA_{com}-Ch/G, (B) CaP_{bm}-Ch/G and (C) CaP_{fs}-Ch/G fabricated via resonance acoustic mixing followed by lyophilisation.

Table 6.6: Elemental percentage (in atomic%) and Ca/P ratios of the cross-linked scaffolds fabricated via resonance acoustic mixing followed by lyophilisation.

Samples name	Elements present in the scaffolds (in atomic %)					Ca/P
	O	Ca	P	Mg	N	
HA_{com}-Ch/G	63.94	8.04	5.19	0	22.83	1.55
CaP_{bm}-Ch/G	63.62	6.94	4.85	0.94	23.64	1.43
CaP_{fs}-Ch/G	71.39	3.11	1.96	0.37	23.18	1.51

6.2.2.5 Functional group analysis by FTIR

FTIR spectra of the crosslinked scaffolds are presented in Figure 6.13 and the peak positions of each scaffold including their corresponding assignments are shown in the Table 6.7.

All the scaffolds showed the characteristic peaks of PO_4^{3-} group (ν_4) related to HA/CaPs at around $550\text{--}611\text{ cm}^{-1}$ [300, 305]. However, lower resolution peaks were observed at this range for the CaP_{fs}-Ch/G. The band at 893 cm^{-1} corresponding to the C-H bending (out-of-plane) for Ch was also identified for this scaffold (CaP_{fs}-Ch/G) [227].

A sharp absorption band ascribed to the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups of HA/CaPs overlapped with C-O stretching of Ch at around 1026 cm^{-1} for all the scaffolds [305, 306]. However, peaks at 1032 and 1062 cm^{-1} were attributed to the C-O stretching of Ch for the CaP_{fs}-Ch/G sample [305, 306]. The C-O-C stretching mode of ether group (C-O-C) at around $1140\text{--}1151\text{ cm}^{-1}$ was correlated to Ch observed for all the scaffolds [309]. However, low intensity of this peak was observed for the CaP_{bm}-Ch/G.

A weak band at around 1300 cm^{-1} corresponded to the C-N stretching of Ch or C-O-C stretching of genipin was identified for all the scaffolds [227].

All the scaffolds showed peaks at around 1377 and 1408 cm^{-1} which were attributed to the C-O-H stretching of Ch and C-N stretching of Ch-genipin interaction, respectively [300].

The characteristic peaks of chitosan corresponded to the N-H bending (amide II) and C=O stretching vibration (amide I) were observed in slightly shifted position from 1654 and 1606 cm^{-1} to 1554 and 1633 cm^{-1} , respectively [227, 300].

In addition, a weak band at around 2800-3000 cm^{-1} and a broad band at around 3000 – 3600 cm^{-1} could be attributed to C-H stretching vibrations (symmetric and asymmetric) and an overlapped of amine and alcohol stretching vibrations of Ch structure, respectively [310].

However, all the bands in between 1300 – 3600 cm^{-1} observed were in low intensity for the $\text{CaP}_{\text{bm}}\text{-Ch/G}$.

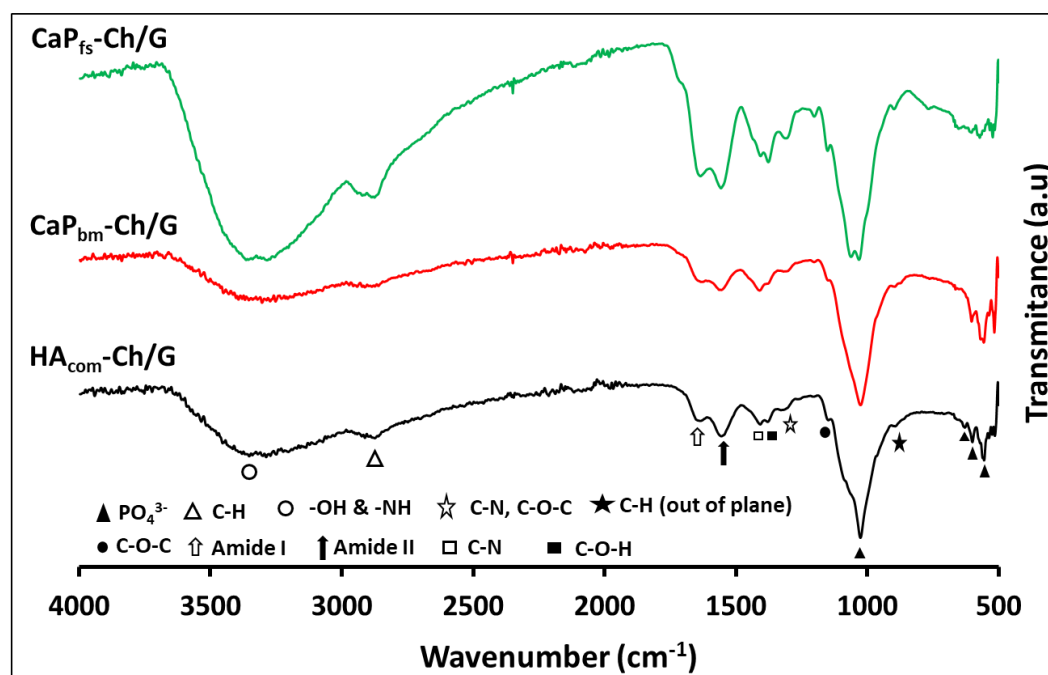


Figure 6.13: FTIR spectra of the scaffolds crosslinked with genipin.

Table 6.7: FTIR peak positions and their corresponding assignments of the cross-linked scaffolds.

Peak positions			Corresponding assignments	References
$\text{HA}_{\text{com}}\text{-Ch/G}$	$\text{CaP}_{\text{bm}}\text{-Ch/G}$	$\text{CaP}_{\text{fs}}\text{-Ch/G}$		
555, 599. 611	557, 603	574, 605	the bending vibration (ν_4) of the phosphate (PO_4^{3-}) groups	[300, 305]
893	871, 893	898	C-H bending Out-of-plane (CH)	[227]
968	-		$\nu_1(\text{PO}_4^{3-})$	[305]
1026	1026	1032, 1062	C-O stretching (CH) / the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups	[305, 306]
1147	1140	1151	(C-O-C) stretching mode	[309]

1311	1302	1309	C-N stretching (CH), C-O-C stretching (G)	[227]
1377, 1408	1377, 1408	1377, 1406	C-O-H stretching (CH), O-H bending (CH), C-N stretching (CH-G)/ the $-CH_3$ bending vibration and the $-CH_2$ symmetrical deformation mode	[300]
1554, 1633	1554, 1633	1558, 1635	N-H bending (CH/CH-G) and C=O stretching (CH-G) or / the characteristic peaks of amide II and amide I (attributed to the axial CQO stretching of the acetamido groups (named amide I)/ C=C stretching (G)	[227, 300]
2875	2860	2868	C-H stretching vibrations (symmetric and asymmetric)	[307]
3340	3340	3281, 3363	O-H and N-H symmetrical vibration	[310]

6.2.2.6 Swelling and mass loss study of the cross-linked scaffolds

The swelling test of the crosslinked scaffolds was performed in PBS for 96 hrs and the data are presented in Figure 6.14. The microsphere containing crosslinked scaffolds (CaP_{fs} -Ch/G) showed the highest water uptake (~981 %) within first 15 mins and then remained almost constant up to 96 hrs (1025%). Whereas the water uptake of 943 % and 471 % were observed for CaP_{bm} -Ch/G and HA_{com} -Ch/G at the same time point of 15 mins. The percentages of mass loss of the scaffolds were measured after 96 hrs immersion in PBS and presented in Figure 6.15. The mass loss of CaP_{fs} -Ch/G and CaP_{bm} -Ch/G were found to be almost same (3.08 % and 2.82% respectively) and the lowest mass loss (1.8 %) was observed for the HA_{com} -Ch/G.

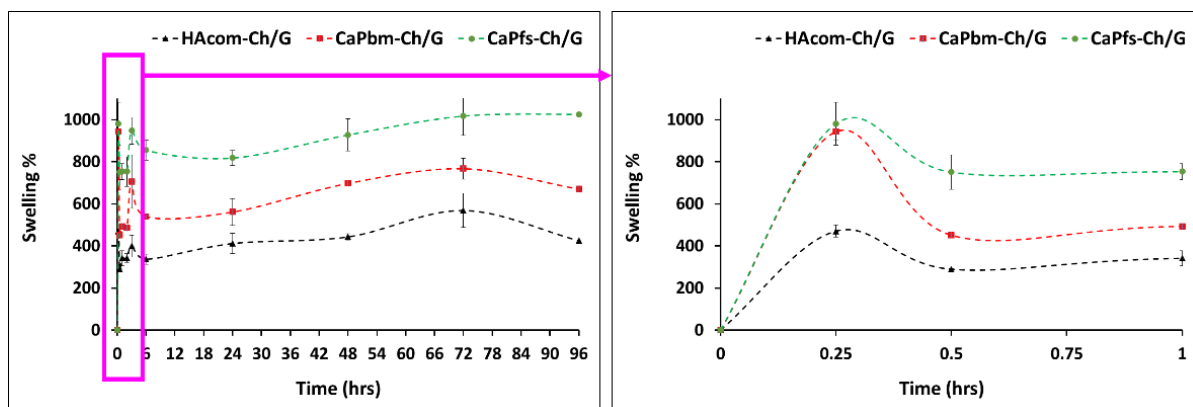


Figure 6.14: Water uptake of three different cross-linked scaffolds fabricated measured in PBS upto 96 hrs (A) and first 1 hr (B).

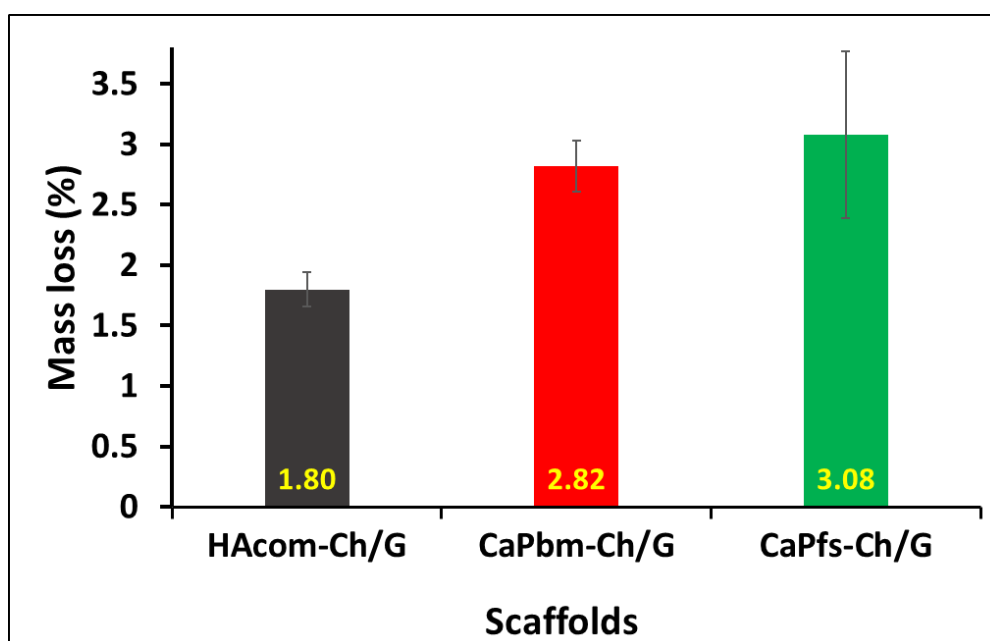


Figure 6.15: Mass losses (%) of the cross-linked scaffolds fabricated after 96 hrs of immersion in PBS and dried overnight at 50 °C.

6.2.2.7 FTIR analysis after swelling test

FTIR study was carried out after swelling test of the crosslinked scaffolds and presented in Figure 6.16. It was observed that the intensity of all the absorbed bands for all three crosslinked scaffolds decreased after 96 hrs immersion in PBS in comparison to the initial samples (Figure 6.13).

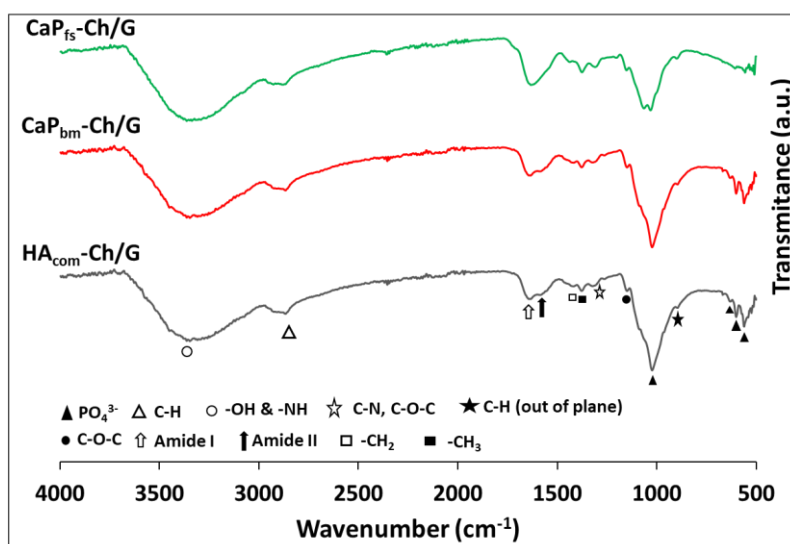


Figure 6.16: FTIR spectra of the cross-linked scaffolds fabricated after 96 hrs of immersion in PBS.

6.2.2.8 Mechanical properties

The results from compression testing of the crosslinked scaffolds (dry) are presented in Figure 6.17. The compressive strength of the cross-linked scaffolds was found to be within the ranges of 8 (for HA_{com}-Ch/G) and 26 kPa (for CaP_{bm}-Ch/G). The highest compressive modulus was also obtained for the CaP_{bm}-Ch/G (0.4 kPa) and lowest for the HA_{com}-Ch/G and CaP_{fs}-Ch/G (0.12 kPa).

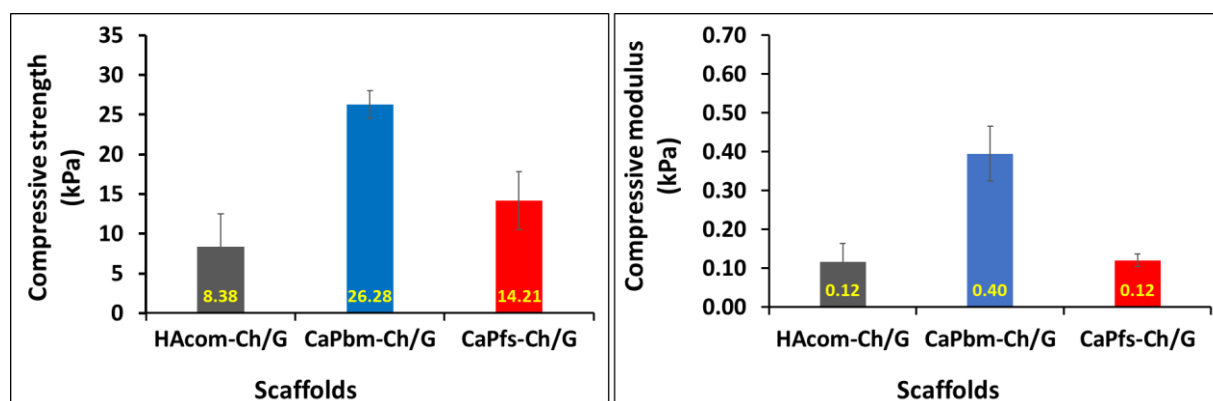


Figure 6.17: Compressive strength (A) and compressive modulus (B) of the crosslinked scaffolds fabricated from commercial chitosan (Ch) and commercial hydroxyapatite (HA) or calcium phosphates (CaPs) obtained from hydrothermal synthesis (ht), ball milling (bm) and flame spheroidisation (fs) and crosslinked with genipin (G).

6.2.3 Films

6.2.3.1 Film Produced

Three different films ((F)HA_{com}-Ch, (F)CaP_{bm}-Ch and (F)CaP_{fs}-Ch) were prepared via solvent casting method initially using commercial HA or CaPs (obtained from eggshells and struvite via ball milling followed by sintering and followed by flame spheroidisation) and commercial chitosan. The images of the films developed are shown in Figure 6.18.

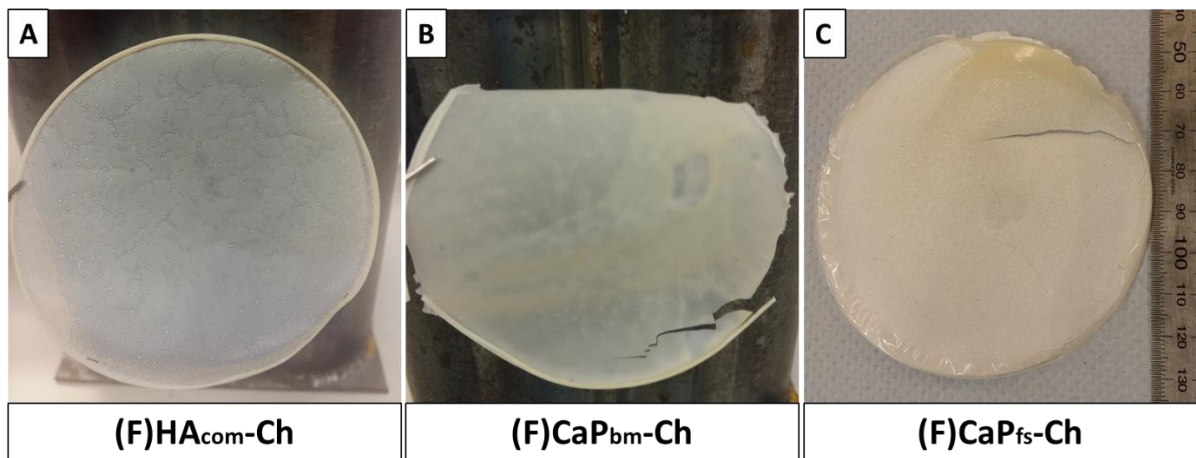


Figure 6.18: Images of films (A) (F)HA_{com}-Ch, (B) (F)CaP_{bm}-Ch and (C) (F)CaP_{fs}-Ch prepared via solvent casting method.

6.2.3.2 Morphology characterisation using FEG-SEM

The surface morphologies of the films are presented in Figure 6.19. A porous structure for the films prepared using commercial hydroxyapatite was observed, whilst non-porous structure was seen for the films prepared using CaPs and chitosan. CaPs particles or microspheres were clearly seen in the films produced (see Figure 6.19B2-C2).

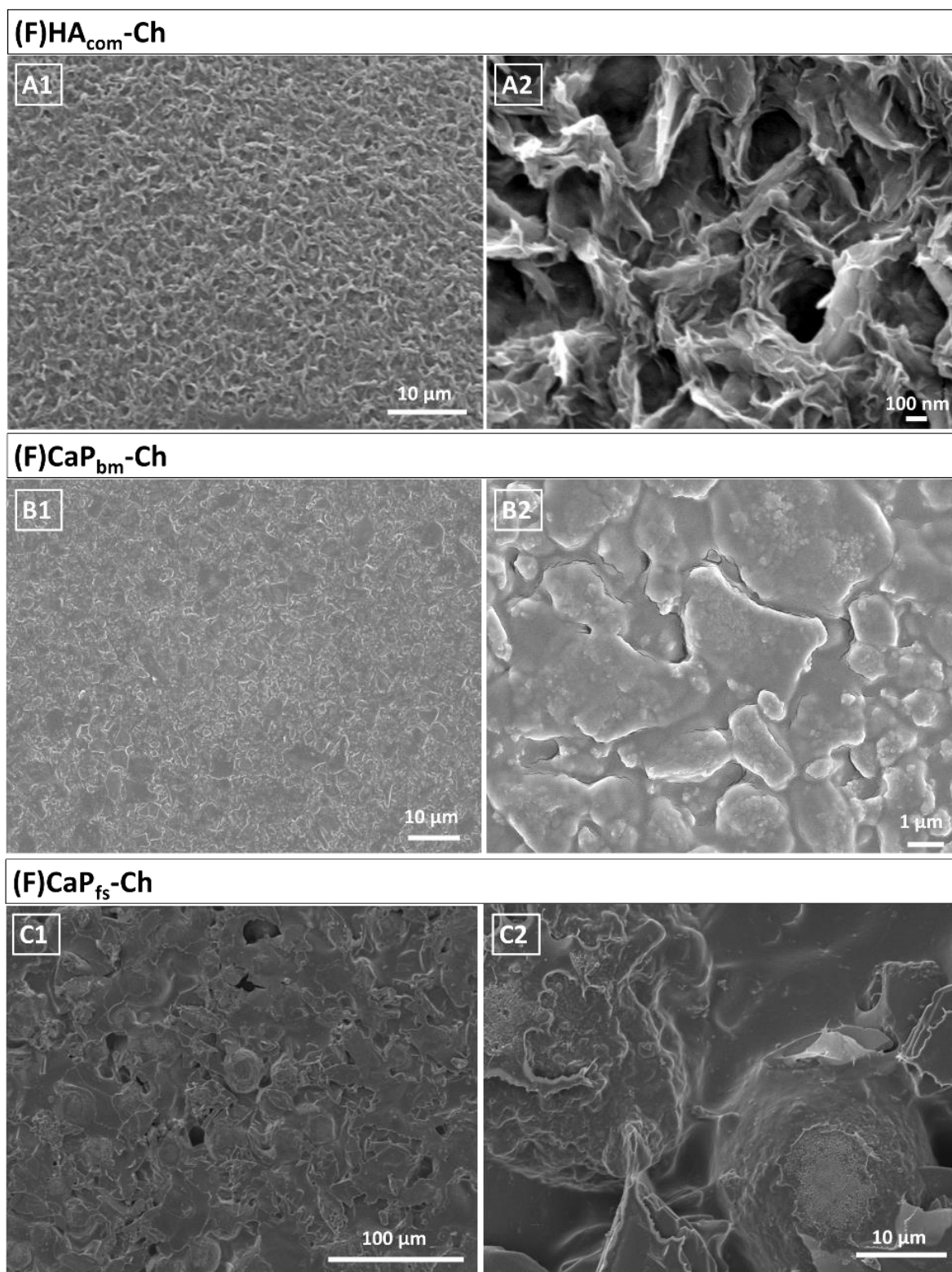


Figure 6.19: The surface morphology of films fabricated via solvent casting method using commercial chitosan and (A) commercially available hydroxyapatite (HA_{com}), B) CaP_{bm} obtained from eggshells and struvite via ball milling followed by sintering and C) CaP_{fs} obtained via ball milling followed by flame spheroidisation.

6.2.3.3 Elemental analysis via EDX

The EDX spectra and the elements present (atomic %) in the films fabricated are presented in Figure 6.20 and Table 6.8, respectively. The corresponding EDX spectrum revealed that the chemical elements of the HA/CaP-Ch films were mainly composed of Ca, P, Mg, O (derived from HA/CaP) and N (derived from Ch). A minor amount of Si was only observed for the (F)CaP_{bm}-Ch. The Ca/P (atomic %) ratios for the commercial HA and synthesised CaPs in the films were found to be in the range of 1.49 (for (F)CaP_{bm}-Ch) -1.62 (for (F)CaP_{fs}-Ch).

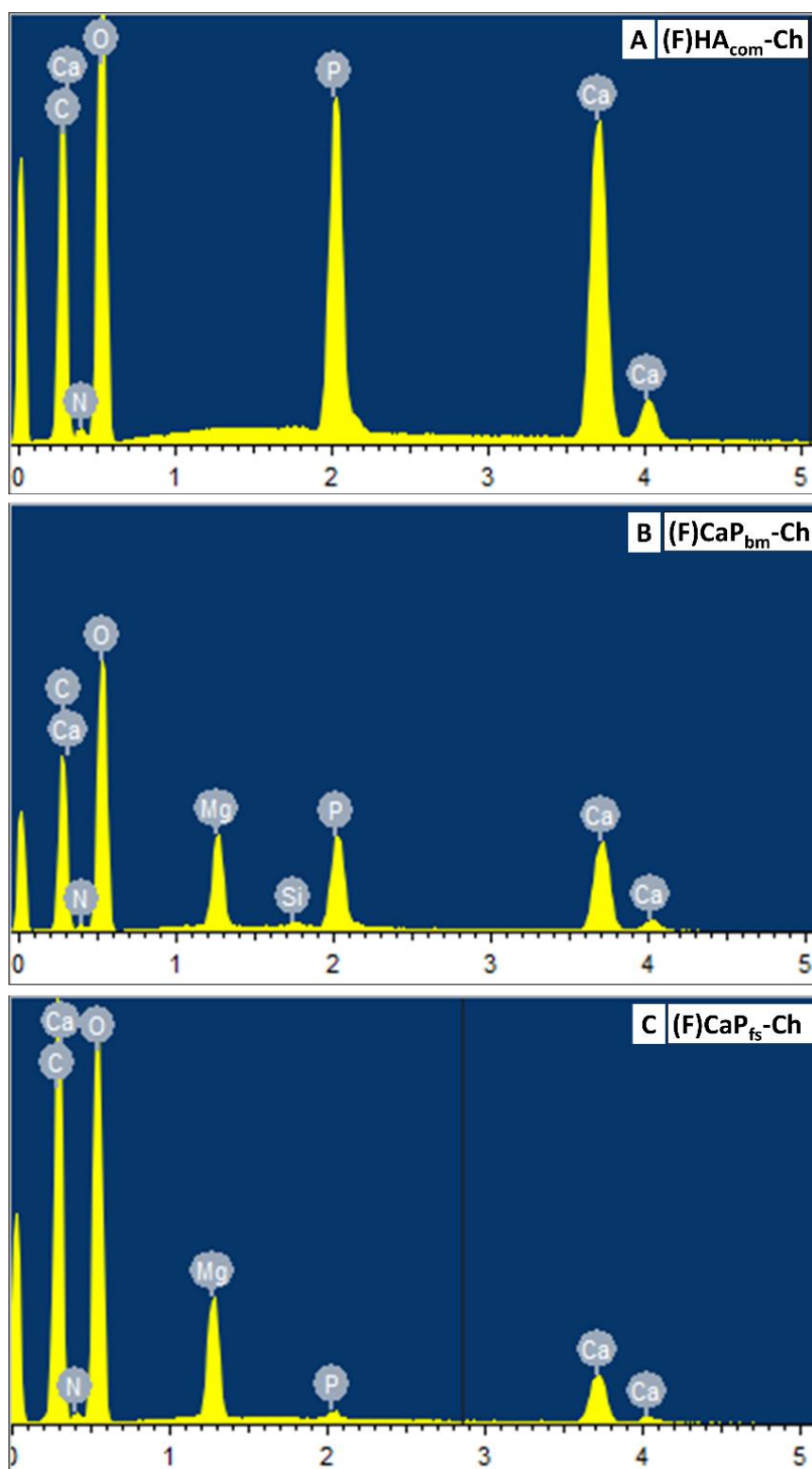


Figure 6.20: EDX spectrum of the films fabricated via solvent casting method using commercial chitosan (Ch) and commercial hydroxyapatite (HA_{com}), CaP_{bm} obtained from eggshells and struvite via ball milling followed by sintering and CaP_{fs} obtained from eggshells and struvite via ball milling followed by flame spheroidisation.

Table 6.8: The elemental percentage (in atomic%) and Ca/P ratios of the films fabricated via casting method using commercial chitosan (Ch) and commercial hydroxyapatite (HA_{com}) and CaPs obtained from eggshells and struvite via ball milling followed by sintering (CaP_{bm}) or followed by flame spheroidisation (CaP_{fs}).

Samples name	Elements present in the films (in atomic %)						Ca/P
	O	Ca	P	Mg	N	Si	
(F) HA_{com} -Ch	63.94	12.42	7.79	0	14.36	-	1.59
(F) CaP_{bm} -Ch	68.99	7.89	5.29	5.89	11.77	0.18	1.49
(F) CaP_{fs} -Ch	74.23	0.65	0.41	7.21	17.50	-	1.62

6.2.3.4 Functional group analysis by FTIR

FTIR spectra of the films are presented in Figure 6.21 and the peak positions of each films including their corresponding assignments are shown in the Table 6.9.

All the films showed the characteristic peaks of PO_4^{3-} group (ν_4) related to HA/CaPs at around $550-611\text{ cm}^{-1}$ [300, 305]. However, lower resolution peaks were observed at this range for the (F) CaP_{fs} -Ch. Peak at 860 cm^{-1} corresponded to carbonated calcium hydroxyapatite, (i.e., PO_4^{3-} substituted by CO_3^{2-}) was observed for the (F) CaP_{fs} -Ch [271].

A sharp absorption peak ascribed to the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups of HA/CaPs at 1010 cm^{-1} was observed for the (F) HA_{com} -Ch and (F) CaP_{bm} -Ch, whereas doublet at 983 and 1024 cm^{-1} for the (F) CaP_{fs} -Ch was observed [305].

A weak band at 1315 cm^{-1} and 1381 cm^{-1} corresponded to the C-N stretching of CH and C-O-H stretching of Ch were identified for the film (F) CaP_{fs} -Ch [300].

Peak at around 1408 cm^{-1} was observed in all the films, which corresponded to the $-CH_2$ symmetrical deformation mode [300].

The peaks for the N-H bending (amide II) and C=O stretching vibration (amide I) at 1550 and 1637 cm^{-1} were observed for the (F) HA_{com} -Ch which corresponded to the characteristic peaks of Ch, on the other hand, only amide II was observed for the (F) CaP_{bm} -Ch and (F) CaP_{fs} -Ch, respectively [300]. In addition, (F) HA_{com} -Ch showed a weak band at around $2800-3000\text{ cm}^{-1}$.

A sharp point at 2868 cm^{-1} and a broad band at around $3000 - 3600\text{ cm}^{-1}$ could be attributed to C-H stretching vibrations (symmetric and asymmetric) and an overlapped of amine and alcohol stretching vibrations of Ch structure, respectively [300, 307]. No such bands for the (F)CaP_{bm}-Ch were observed. However, band at 3240 cm^{-1} for the (F)CaP_{fs}-Ch was only observed [300].

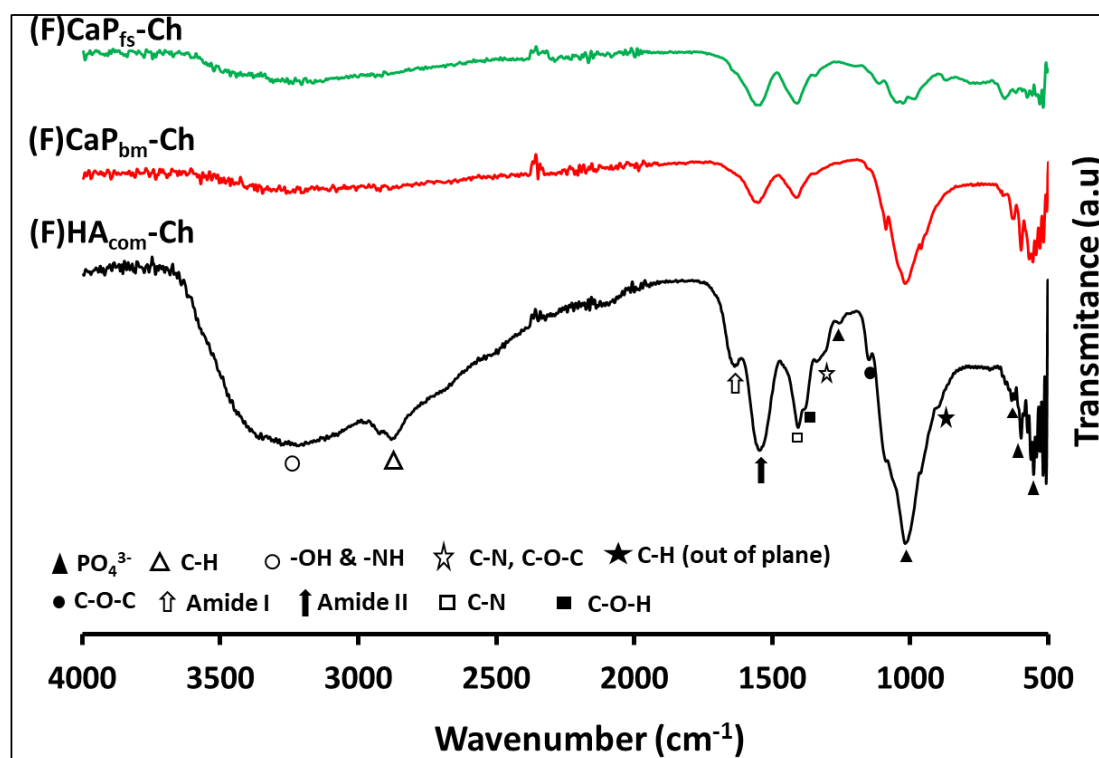


Figure 6.21: FTIR spectra of films fabricated via casting method using commercial chitosan (Ch) and commercial hydroxyapatite (HA_{com}) and CaPs obtained from eggshells and struvite via ball milling followed by sintering (CaP_{bm}) or followed by flame spheroidisation (CaP_{fs}).

Table 6.9: Peak positions and their corresponding assignments for the FTIR spectra of the films.

Sample names/Peak positions			Corresponding assignments	References
(F)HA _{com} -Ch	(F)CaP _{bm} -Ch	(F)CaP _{fs} -Ch		
553-609	555 - 624	657	the bending vibration (ν_4) of the phosphate (PO_4^{3-}) groups	[300, 305]
-	-	870	ν_2 mode corresponds to carbonated calcium hydroxyapatite, i.e., PO_4^{3-} substituted by CO_3^{2-}	[271]
896	896	-	C-H bending (Out-of-plane) (CH)	[308]
962	960	981, 1028	$\nu_1(\text{PO}_4^{3-})$	[305]
-	-	1047	$\nu_3(\text{PO}_4^{3-})$ stretching	[305]
1018	1018	-	C-O stretching (CH) / the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups	[305, 306]
1078	1087	1082		
1122	1130	1111	ether group / pyranose m3 (C-O-C) stretching mode	[305, 306]
1251	-	-	$\nu_3(\text{PO}_4^{3-})$ stretching/ HPO_4^{2-} ions	[273]
1315	-	1338	C-N stretching (CH)	[300]
1381, 1408	1408	1408	the $-\text{CH}_3$ bending vibration and the $-\text{CH}_2$ symmetrical deformation mode, respectively	[300]
1544, 1635	1544	1645	amide II and amide I	[300]
2870	-	-	C-H stretching vibrations (symmetric and asymmetric)	[307]
3300	-	3240	an overlap of amine and alcohol stretching vibrations	[300]
-	-	3456	N-H symmetrical stretch of CH	[300]

6.2.3.5 Swelling and mass loss study of the films

The results of swelling properties of the films performed in PBS up to 96 hrs are presented in Figure 6.22. The microsphere containing films ((F)HA_{com}-Ch) showed the highest water uptake (400 %) within first 15 mins and then remained constant till 96 hrs. Other two films ((F)CaP_{fs}-Ch and (F)CaP_{bm}-Ch) also followed the similar water uptake trends to the (F)HA_{com}-Ch. The highest swelling of 215 % for the (F)CaP_{bm}-Ch and 93 % for the (F)CaP_{fs}-Ch were observed after first 15 mins immersion of films in PBS then gradually decreased to 138 and 56 % respectively, at 6 hrs and then remained almost constant until end of the experiment at 96 hrs.

The percentages of mass loss of the films were measured after 96 hrs immersion in PBS and presented in Figure 6.23. The highest mass loss (34.34 %) for the (F)CaP_{bm}-Ch and the lowest mass loss (6.35 %) for the (F)HA_{com}-Ch were observed. The (F)CaP_{fs}-Ch film showed almost similar mass loss (32.6%) to the (F)CaP_{bm}-Ch.

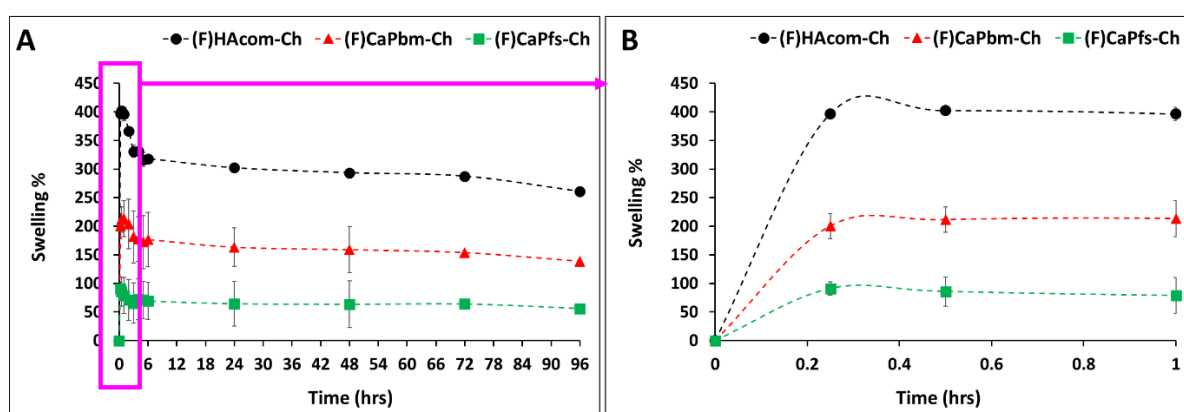


Figure 6.22: Water uptake of three different films fabricated measured in PBS up to 96 hrs (A) and first 1 hr (B).

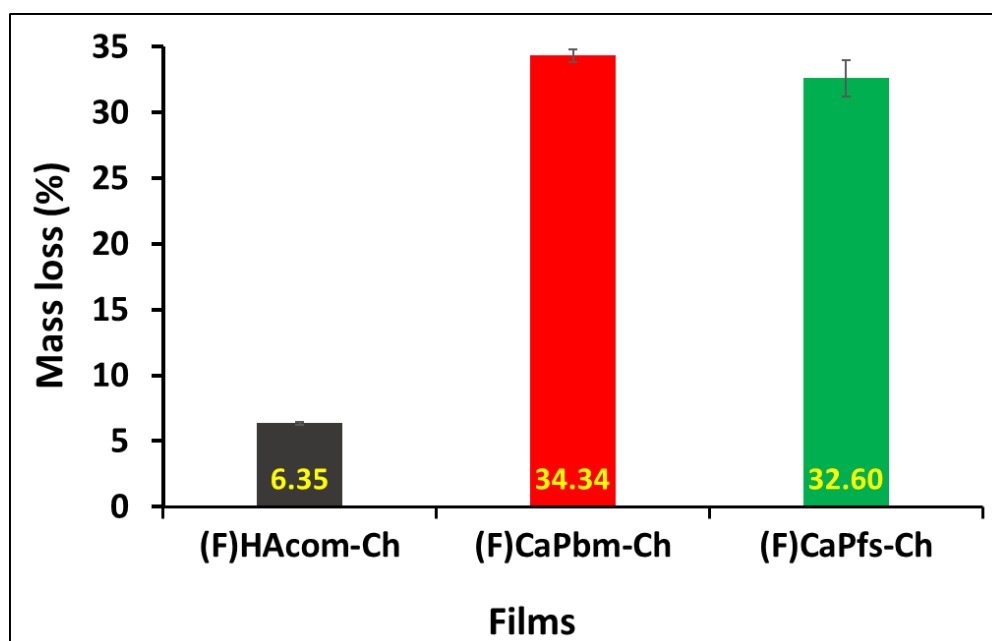


Figure 6.23: The percentages of mass loss of the films fabricated after 96 hrs of immersion in PBS.

6.2.3.6 FTIR analysis after swelling test

FTIR study was carried out after swelling test of the films and presented in the Figure 6.24. The intensity of all the absorbed bands for all three films decreased slightly after swelling 96 hr tests compared to the starting films (before immersion in PBS, see Figure 6.21). This can be clearly seen from Figure 6.24.

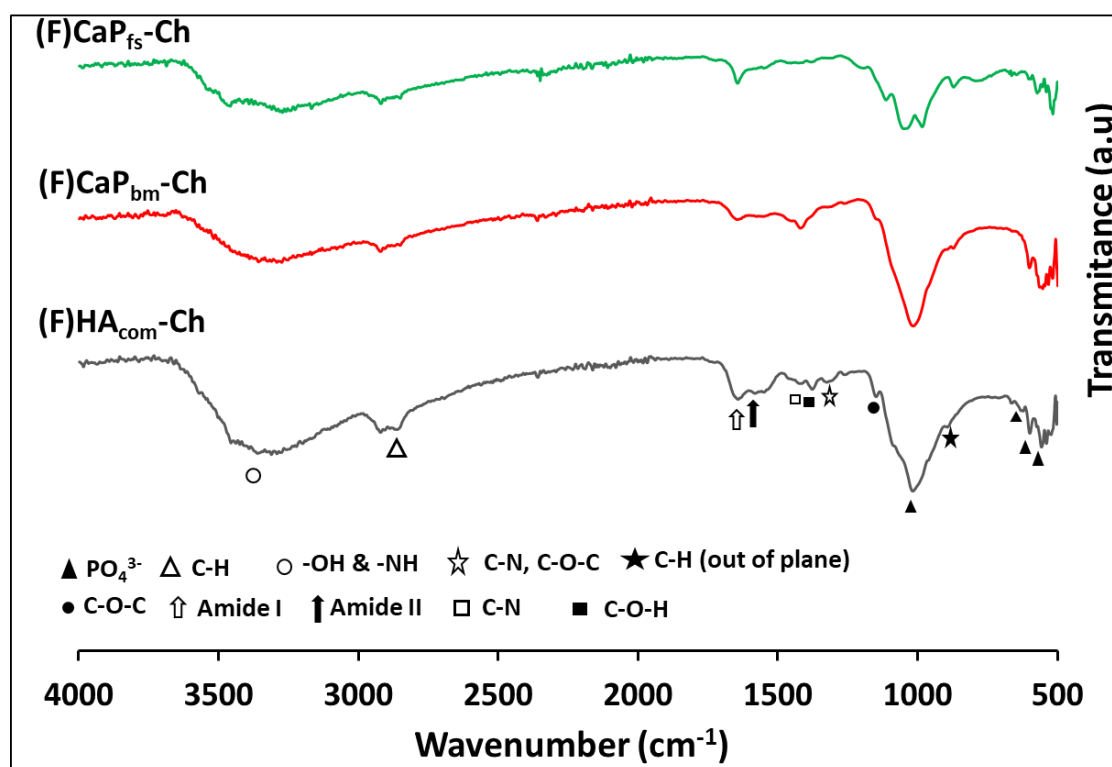


Figure 6.24: FTIR spectra of the films fabricated after 96 hrs of immersion in PBS.

6.3 Discussion

The aim of this study was to prepare and characterise the chitosan (Ch)-based composite scaffolds and films incorporating hydroxyapatite (HA) and/or other calcium phosphates (CaPs) which were prepared using different methods such as hydrothermal synthesis, ball milling followed by sintering and the flame spheroidisation processes.

Ch-HA scaffolds have been widely explored for biomedical applications because of their excellent biocompatibility and bioactivity. HA and nano HA have been explored mostly for bone-related applications. Moreover, Ch has high affinity for HA [305]. A variety of naturally-derived HA (such as from eggshells [217, 311, 312], fish bone etc. [305, 313, 314]) has been used in fabrication of scaffolds. Commercial Ch is a seafood waste-derived product [315-317] and the selected CaPs prepared from waste-derived precursors (eggshells and struvite) via three different methods mentioned above were used to fabricate composite in the form of scaffolds and films aiming for the application in bone tissue engineering [305]. This would also contribute to recycling of the waste materials, leading to sustainable biomaterials.

6.3.1 Scaffolds

6.3.1.1 Morphological analysis

The interconnected pores in the scaffolds fabricated were confirmed by SEM imaging (see Figure 6.3) and this interconnected porous architecture would be essential to support osteogenesis via cell migration, cell proliferation and vascularisation inside the scaffolds [308, 310].

The transverse cross-section of the scaffolds presented a significantly different pore structure compared to the longitudinal cross-section (Figure 6.3). In the transverse cross-section, a layered lamella-like structure with elongated ellipsoid pores was observed for the scaffolds of CaP_{ht}-Ch and CaP_{bm}-Ch, which was similar to the porous structure of pure Ch scaffolds as reported by Pitrolino *et al.* [214]. Also, radially oriented pores were observed for the HA_{com}-Ch and CaP_{fs}-Ch. Whereas, images of longitudinal cross-section showed rounded porous structure for all the scaffolds. EzEldeen *et al.* [227] also observed different pore structure in transverse and longitudinal cross-sections, where the images of transverse cross-section of the scaffolds contained elongated pores or radially oriented lines of pores.

6.3.1.2 Elemental analysis

The corresponding EDX spectrum in Figure 6.4 and elemental analysis in Table 6.3 for the scaffolds prepared revealed that the chemical elements of the Ch-CaP composite scaffolds mainly composed of Ca, P, O, N and Mg, which were derived from HA/CaP and N from Ch. The Ca/P (atomic%) ratios (1.51-1.72) for the HA-CaP scaffolds were almost similar to their respective starting CaPs (see Table 6.3) which confirmed no chemical reaction had occurred (as expected) during the preparation of scaffolds via resonance acoustic mixing followed by freeze drying.

6.3.1.3 FTIR spectrum

The FTIR spectrum of the scaffolds exhibited all the characteristics peaks of Ch and CaPs indicating their presence in the scaffolds (see Figure 6.5). Peak broadening observed in the range of 550-605 cm⁻¹ and 960-1100 cm⁻¹ (correspond to the different vibration modes of

PO_4^{3-}) which confirm the interaction of PO_4^{3-} group of HA with the chitosan [304]. Danylchenko *et al.* [304] also observed significant increase in the width of all the major peaks of hydroxyapatite with the increase in chitosan content, which they attributed to the interaction of chitosan with the respective group of hydroxyapatite. For example, they identified peak broadening at 1050 cm^{-1} due to the interaction of PO_4^{3-} group with the chitosan.

The characteristic peaks of Ch at 1375, 1413, 1587, and 1658 cm^{-1} (corresponded to the $-\text{CH}_3$ bending vibration, the $-\text{CH}_2$ symmetrical deformation mode, amide II and amide I, respectively) were observed shifting towards lower wavenumber for the HA/CaP-Ch scaffolds which suggested the formation of hydrogen bonding between the Ch and HA/CaP [12].

6.3.1.4 Swelling study of the scaffolds

The swelling ability is one of the essential features required for scaffolds to be used in bone tissue engineering, because it evaluated the capability of scaffolds to absorb body fluid for infiltration of cell nutrients and metabolites into the scaffolds. The swelling ratio of the scaffolds increases with the increase of porosity and pore size which would consequently lead to increase in the surface area of the scaffolds that would promote better cell attachment and bone tissue ingrowths [214, 310, 318].

The scaffolds fabricated were tested for the swelling capability for 96 h immersion in PBS and mass loss was also measured after 96 h. All the scaffolds revealed a strong ability to absorb PBS and showed rapid swelling within the first 15 mins except $\text{CaP}_{\text{ht}}\text{-Ch}$, which showed slower PBS uptake before reaching the saturation level (426%) at 6 hrs. The high swelling ability of the scaffolds may be due to the presence of free amino and hydroxyl groups in the Ch matrix [214]. Pitrolino *et al.* also reported similar higher water uptake capability of Ch-HA scaffolds, which was suggested to be due to the hydrophilic nature of Ch [214].

The $\text{CaP}_{\text{fs}}\text{-Ch}$ showed 1.2, 1.9 and 3.5 times higher PBS uptake within first 15 min compared to $\text{HA}_{\text{com}}\text{-Ch}$, $\text{CaP}_{\text{bm}}\text{-Ch}$ and $\text{CaP}_{\text{ht}}\text{-Ch}$, respectively, this was due to the presence of microspheres inside the structure of $\text{CaP}_{\text{fs}}\text{-Ch}$ scaffolds. The larger size of the microspheres ($24 - 58\text{ }\mu\text{m}$) in comparison to other CaPs particles (in nano to submicron range) generally tended to settle at the bottom of the scaffolds during fabrication (which was confirmed via longitudinal image of $\text{CaP}_{\text{fs}}\text{-Ch}$ scaffolds, see Figure 6.25D1). Therefore, they may not be

equally distributed through the scaffolds and as such behaved more like pure chitosan scaffolds at the upper regions. Pallela *et al.* [305] observed rapid swelling of pure chitosan scaffolds compared to the Ch-HA scaffolds they had prepared. This was suggested to be due to the same reason of hydrophilic nature of Ch as mentioned above [214]. However, the swelling properties of CaP_{fs}-Ch scaffolds started to decrease after 15 mins which could be attributed to the progressive release of the microspheres from scaffolds into PBS as a result of the chitosan swelling. This was confirmed via higher mass loss and changes in FTIR spectra after swelling test (see Figure 6.7 and Figure 6.8).

On the other hand, the observation of lower swelling for the CaP_{bm}-Ch and CaP_{ht}-Ch compared to the HA_{com}-Ch might be due to the presence of Mg(OH)₂ and Mg-whitlockite along with HA in the scaffolds network, respectively, which may effect the porosity and also increase the solution pH, resulting less active sites for swelling [319]. Wu *et al.* reported that the incorporation of HA and MgO into silk fibre reinforced silk fibroin scaffolds (SFFHM) significantly reduced swelling ratio compared to pure silk fibroin scaffolds (SF) [319]. They also observed increase in pH of PBS solution from 7.2 to 7.94 after 48 hr for the SFFHM scaffolds due to the formation of OH⁻ by a reaction between MgO and PBS which precipitated on the surface of scaffolds.

Mass loss of scaffolds after swelling test was found to be the highest 35.54% for the CaP_{fs}-Ch and the lowest 3.84 for the CaP_{ht}-Ch. Mass loss of scaffolds indicated their degradation which was also confirmed via FTIR (reduction of absorption bands see Figure 6.8) analysis [305].

6.3.1.5 Mechanical properties of HA–Ch composite scaffolds

The mechanical properties of the scaffolds depend on a range of factors such as concentration of the materials, method of preparation, final porosity of the scaffolds and the testing method used [214]. Compression strength and modulus of the scaffolds were found to be between 5.91 (for CaP_{ht}-Ch) – 26.22 kPa (for CaP_{bm}-Ch) and 0.04 (for HA_{com}-Ch)-0.79 kPa (for CaP_{bm}-Ch), respectively. Pitrolino *et al.* [214] reported similar values of compressive strength (4.81 ± 0.79 kPa) for their hydrated HA-Ch scaffolds which contained 70% nHA in 4% Ch matrix.

Some studies suggested that to create an appropriate environment (*in vivo* or *in vitro*) for the cells to proliferate and differentiate into functional tissue is more necessary than matching

the exact value of the mechanical strength to the native tissue. Moreover, as the properties of a cell-seeded scaffolds change during the colonisation and integration with the surrounding tissue, an initial low compressive strength of the starting material may not be an issue to achieve long term repair [214].

6.3.2 Crosslinking

Crosslinking of the scaffolds (HA_{com}-Ch, CaP_{bm}-Ch and CaP_{fs}-Ch) was performed using naturally derived crosslinker (genipin) aiming to enhance the mechanical properties of the scaffolds. The crosslinked scaffolds were found to be dark in colour, whereas the non-crosslinked scaffolds were white. The colour change of the scaffolds can be attributed to the crosslinking of genipin with the amino groups of chitosan which formed a blueish-violet colour pigment [44, 320]. EzEldeen *et. al.* explained that under acidic and neutral conditions, the amino groups of natural materials/ chitosan attacked the olefinic carbon atom at the C3 position of genipin, resulting in an opening of the dihydropyran ring with the formation of a tertiary amine. A subsequent nucleophilic substitution of the carboxymethyl group (by C11) of genipin with the secondary amino group of Ch had also occurred [227]. EzEldeen *et. al.* [227] also reported that the first reaction can be confirmed by an increase in the intensity of the C-N band around 1,078 cm⁻¹ in the FTIR spectra, while the second reaction can be confirmed by the secondary amide band at 1,546 cm⁻¹ (characteristic of N-H bending) and the peak at 1,633 cm⁻¹, which was assigned to C=O stretching in secondary amides. However, in this study, the characteristic band of C-N related to Ch-genipin crosslinking at 1078 cm⁻¹ was not clearly observed for the crosslinked scaffolds. On the other hand, shifting of the bands corresponding to the N-H bending and C=O stretching in secondary amides was observed at around 1556 and 1637 cm⁻¹, confirming the partial crosslinking of Ch with genipin. Henna *et. al.* [46] also observed band at 1535 cm⁻¹ and suggested that the deformation of N-H with the formation of secondary amide due to the reaction of ester (C-O-C) and O-H groups in genipin with the amino group in Ch. They also found C=O vibration of secondary amides at 1623 cm⁻¹. In addition, the peak at around 1,406 cm⁻¹ was attributed to C-N stretching of Ch-genipin crosslinking and -CH₂ stretching mode of Ch also overlapped. However, the peak at 1,015 cm⁻¹ corresponding to C-OH stretching vibrations of crosslinking Ch-genipin was not observed.

Morphological structure of the crosslinked scaffolds showed reduction in pore sizes (e.g, from 31-169 μm to 6-146 μm for HA_{com}-Ch) which was suggested to be due to the effect of crosslinking agent genipin (see Figure 6.11). Similar observations of pore size reduction for the chitosan-genipin crosslinked scaffold had also been reported by EzEldeen *et al.* [227].

No significant difference in elemental composition for the crosslinked scaffolds compared to the bare scaffolds were observed. However, the Ca/P ratio decreased slightly.

The study on mechanical properties of the crosslinked scaffold revealed that the compressive strength slightly increased for the scaffold containing commercial HA from 6 to 8 kPa; however, mechanical properties remained almost same at 26 and 14 kPa for CaP_{bm}-Ch/G and CaP_{fs}-Ch/G, respectively. This was suggested to be due to the partial cross-linking of scaffolds with genipin which was confirmed from the FTIR spectra (see Figure 6.13).

Swelling properties of the scaffolds increased (18% for HA_{com}-Ch, 311% for CaP_{bm}-Ch, 145% for CaP_{fs}-Ch) after crosslinking (see Figure 6.14), which was unexpected. Therefore, repeat experiments for the crosslinked scaffolds should be required for further confirmation.

The scaffolds degraded over the 96 hr immersion time which was confirmed via mass loss% and via FTIR spectra as decrease in peak intensities was observed, specially peak at 1556 cm^{-1} assigned to amide II was found to have completely disappeared for all the scaffolds after 96 hrs immersion in PBS (see Figure 6.16). However, mass loss of the crosslinked scaffolds was found to be significantly lower compared to non-cross-linked scaffolds which showed the stability of the scaffolds after crosslinking.

6.3.3 Films

Three different films were fabricated using commercial Ch solution and HA/ CaPs obtained via ball milling followed by sintering (CaP_{bm}) and flame spheroidisation (CaP_{fs}) methods. Film containing commercial HA was comparatively transparent and flexible whereas the other two films containing CaP_{bm} and CaP_{fs} were brittle and less transparent (See Figure 6.18). This was probably due to the incorporation of larger particle size of CaPs (ball milled CaPs and microspheres) in the Ch matrix.

Interestingly, the film containing commercial nano HA (HA_{com}-Ch) formed porous structure and HA particles were homogeneously distributed all over the film (as no agglomeration was seen from the SEM image, see Figure 6.19A). On the other hand, CaP_{bm}-Ch film showed non-porous structure and the CaP particles tended to aggregate in a few microns in size (see Figure 6.19B). However, the particles seemed uniformly distributed with the Ch matrix. Porous structures with small pores of the film were also confirmed for the microsphere containing film (see Figure 6.19C). EDX spectra and elemental analysis confirmed the Ch and HA/CaPs due to the presence of N, Ca and P in the films (see Table 6.8). The presence of Ch and HA/CaPs in the films were also confirmed via FTIR. For example, the characteristic bands related to PO₄³⁻ group of HA at around 550-650 cm⁻¹ were confirmed for all the films. However, bands at around 1020 - 1128 cm⁻¹ corresponded to C-O-C and C-O stretching mode of Ch and PO₄³⁻ vibration of HA revealed the overlapping of these bands. Becerra *et al.* also observed overlapping the C-O-C vibration bands of Ch with the PO₄³⁻ vibration bands of HA in the region of 1150–1020 cm⁻¹ for their composite membranes of Ch/HA [321]. Moreover, Ch showed the deformation of N-H at 1587 cm⁻¹, whereas shifting of this band to the lower wavelength was observed at 1545 cm⁻¹ for the (F)HA_{com}-Ch and (F)CaP_{fs}-Ch and at 1550 cm⁻¹ for the (F)CaP_{bm}-Ch. This was due to the interaction between the positively charged amino group (–NH₂) of Ch and PO₄³⁻ of HA [321]. Becerra *et al.* [321] also observed similar shifting of this band from 1545 cm⁻¹ to 1532 cm⁻¹ for the Ch-HA composite [321]. Band related to C=O stretching of amide I at 1658 cm⁻¹ was observed for the Ch, whereas shifting of this peak observed at 1635 cm⁻¹ for the (F)HA_{com}-Ch and disappeared for the (F)CaP_{bm}-Ch and (F)CaP_{fs}-Ch, which might be due to the interaction of the amine on the Ch and PO₄³⁻ in HA/CaPs [322].

The swelling behaviour of the fabricated films were studied to evaluate the water uptake capability of the films due to the various factors such as porosity, presence of hydroxyl and amine groups and hydrophilic nature. The nano-HA containing film ((F)HA_{com}-Ch) showed the maximum swelling behaviour (402%) at the first 30 mins when compared to other films, due to their porous structure (Figure 6.19A2) which provided higher the surface area to absorb PBS. When the water penetrated into the films, the amine and hydroxyl group in Ch tended to form bonding with water and further led to enhanced swelling increasing the porosity [323]. The reduction in peak intensity of FTIR spectra of all the films after 96 hrs immersion in PBS confirmed the degradation of the films. However, all the films remained relatively stable (mass loss 6% for the (F)HA_{com}-Ch and 32-34% for the (F)CaP_{bm}-Ch and (F)CaP_{fs}-Ch) after 96 hrs immersion in PBS. The mechanical test could not be carried out due to the brittleness of the films.

In summary, the scaffolds and films developed using eggshells, struvite and chitosan could be useful for the implementation of bone repair and regeneration treatment. However, further development (i.e. by changing the chitosan and HA/CaPs ratio, increasing immersion time in cross-linking agent) and *in vitro* cell culture assessment would be required to determine their potential for the applications proposed.

CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

This study focused on the utilisation of waste-derived materials (i.e. eggshells as calcium source, struvite as an alternative phosphorus source and acetic acid as a dissolution media) to produce value-added product (i.e. calcium phosphates, CaPs) with a view towards sustainable manufacture of biomaterials. Nano-crystalline CaPs were prepared using continuous flow hydrothermal synthesis and ball milling methods. The CaPs microspheres were subsequently prepared by flame spheroidisation of ball milled samples. By utilising CaPs prepared in this study, composites were fabricated in forms of scaffolds and films via resonance acoustic mixer followed by freeze drying (for scaffolds) or casting (for films).

Different morphologies of nano HA were prepared from eggshells and conventional P source (di-ammonium hydrogen phosphate) by altering the process parameters of continuous flow hydrothermal synthesis method. For examples, mostly a platelet morphology of HA (37 - 331 nm) and a few rod-shaped HA (11 - 41 nm in diameter) formed at 200 °C. Although, at a higher temperature of 350 °C, a mixture of the nanorods (21 – 47 nm dia) and nanoplatelets (37 – 340 nm) was obtained. Interestingly, a tube morphology of HA (32 – 212 nm diameter and 15 – 49 nm inner wall thickness) was produced at 350 °C when NH_4OH was used to adjust pH at 10. Moreover, only rod-shaped HA formed at both temperatures when pH of the precursors was maintained at 10 using NaOH. The Ca/P (atomic%) ratio of nano HA prepared was found to be 1.43-1.63 which indicated the formation of non-stoichiometric HA due to the presence of trace elements such as Si, Mg, Cu obtained from the eggshells. On the other hand, use of struvite as an alternative renewable P source along with either $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ or eggshells as a conventional Ca source, nanocrystalline pure magnesium whitlockite (Mg-WH) or Mg-containing hydroxyapatite (HA) and/or biphasic Mg-WH and HA with various morphologies were formed. For example, a tubular morphology of HA (37–266 nm diameter) along with rhombohedral Mg-WH (96–214 nm) were produced at a higher temperature (350 °C), while an ellipsoidal-shaped particles (96–273 nm) of HA were formed at 200 °C using acetic acid as a dissolution media for waste-derived precursors. The formation of Mg-WH was due to the presence of Mg in struvite. Mg-WH could potentially be used for the application of bone

treatment as it is occupying approximately 25–35 wt% of the inorganic portion of human bone.

To minimise the usage of harmful synthetic chemicals, such nitric acid, acetic acid, ammonium hydroxide, sodium hydroxide etc; the ball milling method was conducted for 2 hrs at 400 rpm to synthesise CaPs using waste-derived precursors of raw eggshells or calcined eggshell powders and struvite. The HA along with brucite [$\text{Mg}(\text{OH})_2$] were formed by simple ball-milling of calcined eggshell powder and struvite. However, higher crystalline biphasic CaPs (HA and Mg-WH) were obtained upon heat treatment of ball-milled raw eggshell-struvite for 3 hr at 1150 °C, following the production of multiphase CaPs (stanfieldite as a main phase along with HA and Mg-WH) at 900 °C and the Farringtonite ($\text{Mg}_3(\text{PO}_4)_2$) as a predominant phase at 700 °C. Whereas, the formation of higher crystalline biphasic CaPs (HA and Mg-WH) was observed at both 900 and 1150 °C from the ball milled calcined eggshell-struvite, following the production of higher crystalline HA at 700 °C.

To manufacture CaPs microspheres, the ball-milled samples of two different size ranges (i.e. 63–100 μm and 100–160 μm) of granules were flame-spheroidised using oxy-acetylene gas (gas ratio of 3:3). The α -TCP was found to be formed as predominant phase among the CaPs microspheres produced due to the high temperature of the flame. Microspheres of pure α -TCP (along with MgO) (29.8 – 54.55 μm) and multiphasic CaPs (HA, α -TCP, Stanfieldite and Mg-WH) (42.1 – 107.64 μm) were obtained from a smaller size range (60–100 μm) and the larger granules (100–160 μm) of ball-milled raw eggshell-struvite samples, respectively. Whilst only biphasic CaPs microspheres (α -TCP and HA) were produced from both size ranges of ball-milled calcined eggshell-struvite granules. The Ca/P ratio of the microspheres were found to vary between 1.5 and 1.65.

Aiming to prepare a composite entirely from waste-derived materials for bone tissue engineering, the calcium phosphate-chitosan (CaP-Ch) scaffolds were fabricated via a freeze-drying method incorporating commercial HA and/or other CaPs particles and microspheres which were prepared in this study. Resonant acoustic mixer (RAM), a new mixing technology, was used to prepare a homogeneous composite suspension prior to freeze-drying. These scaffolds showed a strong ability to absorb PBS and rapid swelling was occurred within the first 15 mins for all the scaffolds (222 – 418 %) except the one prepared using hydrothermally synthesised CaPs. However, low compressive strength (5–26 kPa) and modulus (0.2–0.8 kPa)

were found. A reduction in porous structure and mass loss was observed when naturally derived crosslinker (genipin) was used, however, no considerable change in mechanical properties was observed, suggesting partial crosslinking of the scaffolds.

Three different composites in a film form were also fabricated using commercial HA and CaPs synthesised via ball milling followed by sintering and flame spheroidisation by casting the composite suspension prepared using RAM. The films containing commercial HA exhibited highly porous structure with higher swelling properties (400%) and lower mass loss (6.4 %). Whilst the composite films containing ball milled CaPs possessed non-porous structure and showed a moderate range of swelling percentage (211 %) with highest mass loss (about 34 %). However, further development and characterisations will be required for composites (scaffolds and films) to achieve desirable properties.

The outcomes of this study indicated that struvite could potentially be a useful P source for the preparation of CaP-based biomaterials with minimal usage of synthetic chemicals. However, bioactivity characterisation, *in vitro* and *in vivo* studies would be essential to confirm the efficacy of these biowaste-derived biomaterials to be used in biomedical applications.

7.2 Suggestions for future work

Based on the development of this work, the following suggestions have been made for future studies:

- In-depth optimisation of the hydrothermal synthesis process would be required to achieve a better yield and control on the morphology and structure of the produced CaPs.
- Quantitative compositional analysis of the precursor solutions and the product suspensions using ICP-MS to generate a clear understanding of the formation process of CaPs via hydrothermal synthesis process based on the concentration of ions present in solutions before and after the synthesis.
- Determination of the crystallographic parameters of all samples prepared by Rietveld refinement to get more accurate value.

- Particle size distribution analysis of the samples synthesised via hydrothermal synthesis / ball-milling followed by sintering could be carried out via Dynamic Light Scattering (DLS).
- Laser diffraction could be also used to determine the particle size distribution of the microspheres.
- More optimisation of the composite fabrication using resonance acoustic mixing would be required to achieve the optimum mixing conditions and ultimately improved properties of the composites.
- Porosity measurement of the scaffolds prepared would be determined by the Archimedes method and Micro-CT.
- The pore size of the scaffolds would be determined using PenTabletDrive software by measuring the diameter of a minimum of 25 pores in different areas of scaffolds from SEM images and via Micro-CT.
- To evaluate the morphological changes of composites (scaffolds and films) after swelling test, morphology analysis could be carried out via Environmental Scanning Electron Microscopy (ESEM).
- Ion release of both scaffolds/films and cross-linked scaffolds during the swelling test could be measured via ICP-MS.
- The prepared crosslinked scaffolds did not show better properties due to the lack of proper cross-linking. Therefore, the immersion time for the scaffolds in genipin solution could be increased to improve their properties (such as stability, better mechanical properties, lower swelling ratio etc.). In addition, mixing of genipin directly with the composite solution before drying would be another option to achieve a higher degree of crosslinking.
- *In vitro* bioactivity of CaPs samples prepared (particles, microspheres and composites developed using eggshells and struvite) could be carried out by immersion the samples in simulated body fluid (SBF) under a controlled environment of 37 °C up to 28 days. The standard procedure of BS ISO 23317:2014 needs to follow in preparing SBF. A unified method (TC04 method) in *in vitro* evaluation for the apatite-forming ability of CaPs particles/microspheres could be followed, where 75 mg of CaPs particles/microspheres would be immersed in 50 ml SBF solution at 37 °C in a

polyethylene vial and agitated at 120 rpm. At each time point, the CaPs particles/microspheres would be filtered and washed with deionised water and then dried overnight at 50 °C in an oven. XRD, SEM and EDX analysis would be conducted to explore the structural, morphological and compositional changes, respectively. The pH values of the SBF solution would also be measured at each time point. Ion release of samples in SBF at each time point would be conducted via ICP-MS

- Further *in vitro* cell culture and *in vivo* pre-clinical trials for the CaPs prepared would be required to evaluate the bioactivity, cytotoxicity and cytocompatibility of the samples prepared (particles, microspheres and composites developed using eggshells and struvite).

In vitro cell culture could be conducted via the following procedures.

- Sterilisation of CaPs particles/microspheres would be performed throughout two washes of 15 min with ethanol 70% followed by complete evaporation at room temperature in sterile conditions.
- Direct and indirect cell culture and differentiation would be carried out using Immortalised bone marrow-derived human mesenchymal stem cells (MSCs) labelled with the green fluorescent protein. For both indirect and direct cultures, 100 µl of sterile distilled water would be added to each sample followed by three freezing-thawing cycles. Then, 95 µl of sample suspension would be transferred to a clear bottom 96-well plate. Measurement of fluorescence would be performed in an Infinite 200 microplate reader (Tecould, CH) at 480 nm and 520 nm as excitation and emission wavelengths, respectively.
- The scaffold would be seeded with stem cells at a cell density of 1 Million cells per mL.
- Ion release of CaPs particles/microspheres and composites (scaffolds/films) in Dulbecco's Modified Eagle Medium (DMEM) media would also be investigated via ICP-MS in order to reflect the ion release profiles more accurately that the cells may experience within a cell culture environment. The medium would be refreshed every 48 h to perform the cell culture study and values would be recorded at different time points such as at day 1, 2, 6, 10, 12 and 14.

- Metabolic activity assay would be conducted using a PrestoBlue kit according to the manufacturer's instruction.
- Pico-Green® dsDNA quantitation kit would be used to evaluate the effect of the conditioned media on cell growth by measuring DNA amounts in cultures at different time points. The cells would be lysed using a freeze/thaw method before analysing for DNA content.
- To investigate the effect of samples ion extracts on osteogenic differentiation, Alkaline Phosphatase (ALP) activity would be assayed at different time point of culture. Cells would be first washed twice with PBS and 300 µl of assay solution containing 1 mg/ml p-nitrophenyl phosphate and 0.2 M TRIS buffer (SIGMAFAST, Sigma-Aldrich, UK) prepared according to the manufacturer's instructions would be added to each well. ALP activity would be monitored at 405 nm using an Infinite 200 microplate reader (Tecould, CH) pre-set at 37 °C.
- Alizarin Red S staining would be performed to mark mineralised extracellular matrix. Cells would be fixed for 10 min with 4% paraformaldehyde at 4 °C, followed by two PBS washes. Before staining, two washes with deionized water would be performed followed by the addition of 200 µl of 1% w/v Alizarin Red S solution (Sigma-Aldrich, UK). After 15 min, samples would extensively be washed with deionized water before imaging. For stain quantitation, samples would be incubated with 200 µl of destaining solution (20% methanol, 10% acetic acid in deionized water) during 15–20 min before measuring the absorbance at 405 nm.
- Bright-field and fluorescence images of living cells would be taken using an EclipseT2 Nikon microscope coupled with a D3300 Nikon camera. To evaluate the morphological response to the different cells-particles/microsphere aggregates images would be captured through Environmental Scouldning Electron Microscopy (ESEM).
- Three independent repeat study would be performed for biological study and presented as mean ± standard error of the mean (SEM). One-way ANOVA with Tukey's multiple comparison post hoc test would be used. Statistical analysis would be performed with the GraphPad PRISM 7.01 software package.

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