



Review article

Extraction of natural hydroxyapatite for biomedical applications—A review

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ABSTRACT

Hydroxyapatite has recently played a crucial role in the sustainable development of biomedical applications. Publications related to hydroxyapatite as filler for biopolymers have exhibited an increasing trend due to the expanding research output. Based on the latest publications, the authors reviewed the research trends regarding hydroxyapatite use in biomedical applications. Analysis of the Scopus database using the keywords ‘hydroxyapatite’ and ‘biomedical applications’ determined that 1,714 papers were produced between 2012 and 2021. The number of publications related to these keywords more than doubled between 2012 (99) and 2021 (247). The hydrothermal method, solid-state reactions, the sol-gel process, emulsion, micro-emulsion, and mostly chemical precipitation were used to produce synthetic hydroxyapatite. Meanwhile, calcination, alkaline hydrolysis, precipitation, hydrothermal, and a combination of these techniques were used in producing natural hydroxyapatite. Studies in the current literature reveal that shell-based animal sources have been frequently used as hydroxyapatite resources during investigations concerning biomedical applications, while calcination was the extraction method most often applied. Essential trace elements of fish bone, oyster shell, and eggshell were also found in hydroxyapatite powder. Abalone mussel shell and eggshell showed Ca/P ratios closer to the stoichiometric ratio due to the use of effective extraction methods such as manipulating aging time or stirring process parameters. This review should greatly assist by offering scientific insights to support all the recommended future research works, not only that associated with biomedical applications.

1. Introduction

Increasing importance is being placed on a hydroxyapatite-derived scaffold for bone tissue regeneration applications as an alternative to bone grafts [1, 2, 3, 4, 5]. Insufficient worldwide donors [6] and the potential risk of disease transmission [7] affirm that autograft and allograft are not sustainable approaches to bone substitutes. Furthermore, hydroxyapatite has biological similarities [8, 9, 10, 11] to bone tissue, as well as being abundantly available and offering an environmentally friendly solution in biomedical and tissue engineering research [12]. Waste from animal sources such as mammalian bone [13, 14, 15], fish bone and scale [16, 17, 18], and shells [19, 20, 21] play an important role in developing artificial substitutes to address bone defects through hydroxyapatite extraction. The consistently increasing trend of publications in hydroxyapatite-based materials for biomedical applications research over the past decade (2012–2021) also reflects the expansion of worldwide interest in this issue (Figure 1). According to the Scopus database,

the number of publications related to hydroxyapatite and “biomedical applications” more than doubled between 2012 (99) and 2021 (247) (Figure 1). Articles were the most frequently published document type, which accounted for 75.44% (1293 documents) of the total publications. This was followed far behind by Conference Papers and Reviews, with 9.45% (162 documents) and 9.04% (155 documents), respectively. Book Chapters accounted for 4.38% (75 documents). The remaining document publication types (Conference Reviews, Books, Errat, Editorials, Notes, and Retracted) covered less than 2.00% of the total publications. This trend indicates that research into hydroxyapatite, especially in relation to biomedical applications, is still developing and attracting growing attention in the scientific community. The regenerative medicine approach has become a widely recognized topic in recent decades, especially regarding hydroxyapatite [22, 23, 24, 25]. Natural and synthetic hydroxyapatite are among the most common materials currently discussed in the context of bone tissue regeneration, due to their bioactivity, low-cost, non-toxicity, and compatibility with the available

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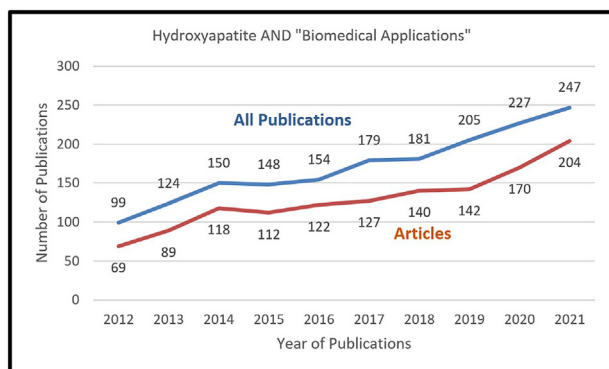


Figure 1. Published articles using keywords “hydroxyapatite” and “biomedical applications” (2012–2021).

applications [26, 27, 28, 29]. Thus, the aim of this review is to highlight ideas and insights from the recent works related to natural hydroxyapatite for biomedical applications. An investigation of published articles in the Scopus database revealed that when searching for articles with the combined keywords of “hydroxyapatite” and “biomedical applications”, 1,714 articles were found to have been published between 2012 and 2021.

2. Hydroxyapatite in brief

Hydroxyapatite has a similar chemical structure and similar properties to the inorganic constituents of bones and teeth. In 1920, Albee and Morrison [30] investigated the influence of calcium phosphates in stimulating bone regeneration for rabbit bone defects. They found that calcium phosphates showed promising potential in bone formation. This discovery paved the way for other researchers to explore and further improve this new field of knowledge such as Haldeman and Moore [31] and Huggins et al. [32].

In their experiment, Haldeman and Moore [31] used monocalcium phosphate, tricalcium phosphate, dicalcium phosphate, and calcium glycerophosphate to observe the efficacy of those inorganic and organic compounds in the union of the rabbit radius bone. They found that the salt quantity did not influence the bone healing rate. The presence of tricalcium phosphate aided the bone formation process more than the other organic and inorganic compounds, which showed no positive influence in the study.

In their work, Huggins et al. [32] observed that bone transplants in dogs to replace the ribs and skull failed to excite ossification. However, the transplantation of primary teeth into the abdominal muscle stimulated the formation of new bone. The finding stimulated further research, such as the use of plaster as a bone substitute [33], the stimulation of alkaline phosphatase activity and cartilage in the tooth matrix [34], bone transplants and implants [35], and bone-grafting materials [36].

Synthetic hydroxyapatite was first introduced during the two world war in the first half of twentieth century by Barrett et al. [37]. It exhibited similar x-ray diffraction patterns and chemical composition to natural bone. In 1993, Basle et al. [38] scrutinized the effects of synthetic and natural hydroxyapatite derived from bovine bone on cellular responses in rabbit bone. It was revealed that bone formation using natural hydroxyapatite as an implant was faster than synthetic hydroxyapatite, indicating a greater osteoblast function. The trend in synthesizing hydroxyapatite from natural sources has continued to the present day, using not only bovine bone but also other mammalian bones, fish bone and scale, shells, plants and mineral sources.

3. Natural hydroxyapatite compared to synthetic hydroxyapatite

Hydroxyapatite is composed of the inorganic mineral components of hard tissues, for instance, the spine [39, 40, 41], skull [42, 43, 44], and teeth [45, 46, 47, 48]. Commercial synthetic hydroxyapatite powders can

be obtained using the hydrothermal method, solid-state reactions, the sol-gel process, emulsion, microemulsion, and mostly chemical precipitation due to the simplicity and cost-effectiveness of these approaches [49]. However, the resulting material may lack various important ions, such as magnesium, sodium, potassium, silicon, strontium, and iron [50, 51, 52, 53]. Table 1 summarizes the biological effects of different trace elements identified in previous studies [54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79]. The ready availability of important ions in xenograft, such as from mammalian bone, or fish bone and scale, as well as its low processing cost, makes it a preferred option for biomedical applications research [80].

Concurrently, shells are also regarded as valuable calcium resources, to which can be added a phosphate precursor to produce hydroxyapatite due to its abundant availability and economic feasibility [81]. Natural hydroxyapatite is free from contamination, has high crystallinity, and is environmentally friendly [82, 83, 84]. A previous study revealed that synthetic hydroxyapatite is far less biodegradable than tricalcium phosphate and natural hydroxyapatite [85]. The superiority of hydroxyapatite from natural origins makes it more desirable for biomedical applications. Figure 2 depicts the differences between hydroxyapatite derived from natural sources and chemically synthesized hydroxyapatite in terms of cost, ca/p ratio, source, trace elements, and processing time. The following subchapter discusses the extraction/synthesis and *in vitro* evaluation of natural hydroxyapatite from different animal sources, based on the latest publications from the Scopus database. Figure 3 illustrates a summary of the origins and synthesis methodology of natural hydroxyapatite from animal sources.

3.1. Biomaterials as natural hydroxyapatite source for biomedical applications

Animal by-product sources from mammalian bones such as porcine, bovine, and ostrich, fish bone and scale, as well as shells (eggs and marine), are primarily comprised of organic and inorganic material. They have been widely utilized as sources of natural hydroxyapatite [86, 87, 88, 89]. Natural hydroxyapatite formation based on these types of biomaterial sources usually involves the process of eliminating organic matter from the mineral matrix to obtain hydroxyapatite directly. Several common methodologies can be employed to extract hydroxyapatite from this group of biomaterials, including calcination, alkaline hydrolysis, precipitation, hydrothermal, and a combination of these techniques (Table 2). The hydroxyapatite extracted from animal by-products has

Table 1. Summary of biological effects of trace elements.

Trace Element	Biological Effects	References
Zinc	Inhibit osteoclast cell formation	[54, 55]
	Avoid osteoporosis	[56]
	Increase angiogenesis	[57]
	Improve the differentiation of osteogenic cells	[58, 60, 61, 62, 63]
Magnesium	Cell adhesion and enhance bioactivity	[64, 65, 66]
	Stimulate cell differentiation	[67]
Fluoride	Stimulate osteoblast activity	[68]
	Avoid osteoporosis	[69]
	Hinder osteoclast proliferation	[70]
	Enhance strength and corrosion resistance	[71]
Silicon	Enhance cell differentiation	[72, 73]
	Improve osteogenic differentiation	[74, 75]
	Enhance mechanical property	[76]
Strontium	Absence of cytotoxicity	[77]
	Enhance osteoblast activity and proliferation	[78]
	Promote cell adhesion, proliferation, and alkaline phosphatase activity	[79]

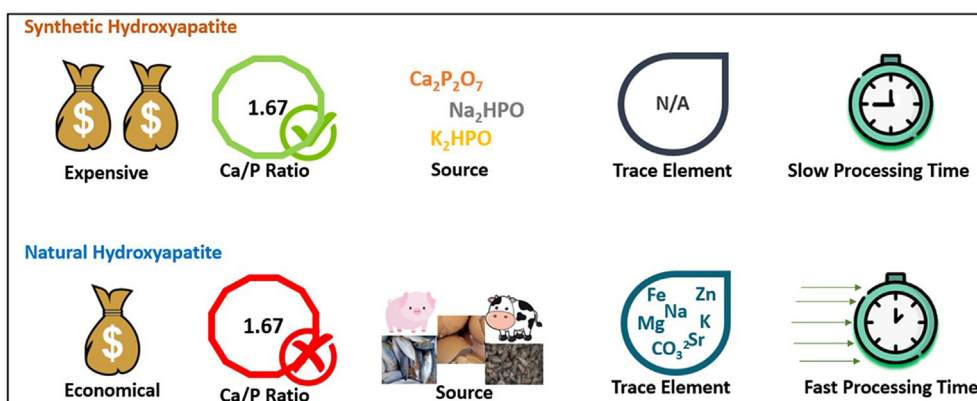


Figure 2. Natural hydroxyapatite vs synthetic hydroxyapatite.

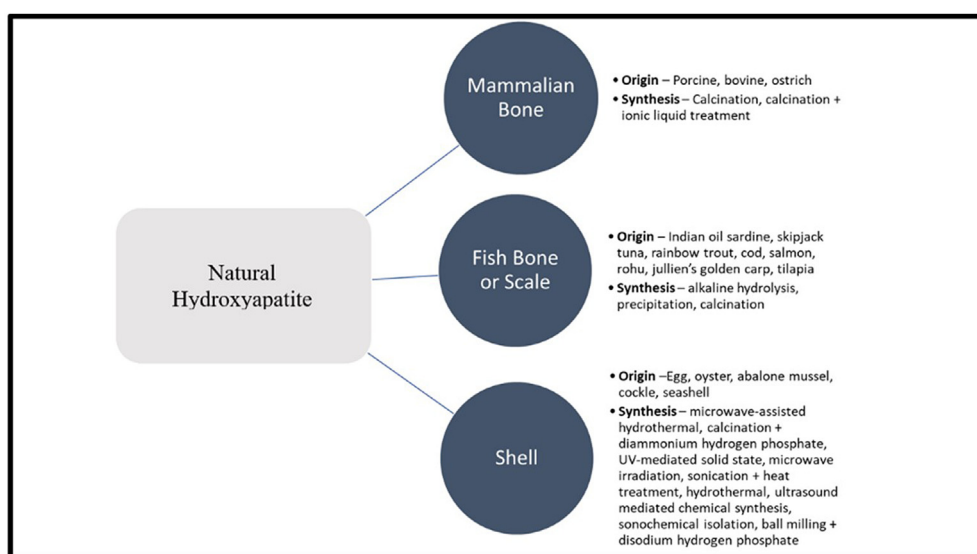


Figure 3. Origins and synthesis methodology of natural hydroxyapatite.

promising potential for various biomedical applications owing to its biocompatible and bioactive properties. Figure 4 displays the most common biomedical applications of hydroxyapatite. Hydroxyapatite was the fundamental material used in bone tissue regeneration and dentistry due to their biological similarity in natural mineral component.

3.1.1. Mammalian bone

Mammalian bones - for instance, porcine [90], bovine [91, 92], and ostrich [93]—have been used as hydroxyapatite sources as they are rich in calcium phosphate. During the time period investigated, bovine bone was reported twice, while porcine and ostrich were reported once each. The Ca/P ratio, crystalline phase, particle size, and shape were reviewed (Table 2). According to the literature, the mammalian bone was pre-treated by boiling for 0.5–4 h prior to the extraction of hydroxyapatite. The lengths of time varied between porcine (0.5 h), bovine (1 h and 3 h), ostrich (4 h) bone. Acetone was sometimes used after boiling to remove invisible fat from the bone [91, 93]. Across the literature, hydroxyapatite was extracted using calcination temperatures between 700 °C and 950 °C, with one study combining the calcination process with ionic liquid treatment [90]. Table 3 summarizes the pretreatment and calcination parameters used in processing mammalian bone.

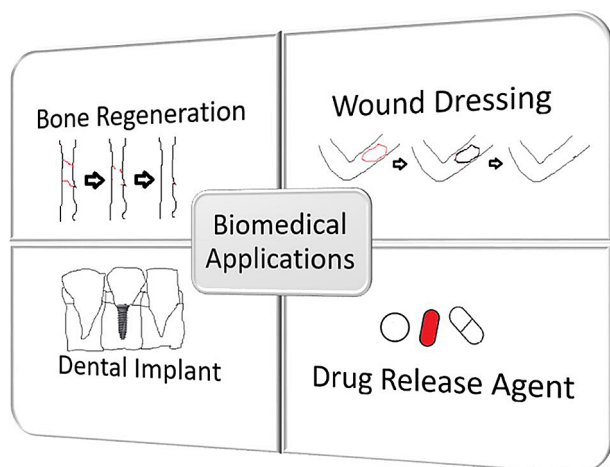
Malla and colleagues used calcination to extract hydroxyapatite from ostrich bone. The extracted hydroxyapatite was heated to 650 °C for 6 h, resulting in plate-like hydroxyapatite. After recalcination at 950 °C, hydroxyapatite particles of irregular shapes and sizes (rod, spherical,

hexagonal, platelet) were detected, most likely due to the grinding process during the sample preparation (Figure 5). It was observed that ostrich bone calcined at 650 °C was free from organic compounds, signifying the effectiveness of the applied thermal decomposition process. Furthermore, the hydroxyapatite crystallinity would be enhanced without the presence of organic compounds. The authors proved that ostrich bone could be a good source of natural hydroxyapatite, based on the physicochemical property testing for non-load bearing applications [93]. The investigation concluded that dwell time and treatment temperature influence the composition of the synthesized powder.

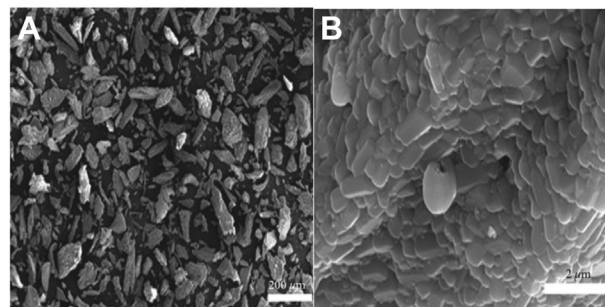
Meanwhile, Liu et al. extracted hydroxyapatite from porcine bone using calcination at 800 °C for 2 h, followed by immersion in sodium fluoride aqueous solution and another calcination at 700 °C for 3 h. The cell proliferation, osteoblastic differentiation, biocompatibility and osteogenic capacity were inspected. Cyclohexane was used as the oil phase to isolate the nanosized particles. The authors used fluorinated porcine bone to observe the biocompatibility and osteogenic capacity of the hydroxyapatite. The presence of fluorine significantly enhanced the osteogenic capacity of the hydroxyapatite [90], based on the *in vitro* and *in vivo* test results. Through quantitative analysis, the volume of new bone tissue generated using fluorinated hydroxyapatite was larger than had been generated with unfluorinated hydroxyapatite, at $39.47 \pm 7.37\%$ and $29.03 \pm 1.70\%$, respectively. It was also revealed through the calvarial defect implant assessment that fluorinated hydroxyapatite exhibited notably better new bone formation activities.

Table 2. Methods of synthesizing hydroxyapatite.

Source	Synthesis/Extraction Method	Ca/P Ratio	Crystalline Phase	Particle Size	Shape	References
Porcine Bone	Calcination + Ionic Liquid Treatment	1.49–1.54	HAP (800 °C)	0.2–0.6 μm	Rod-like	[90]
Ostrich Bone	Calcination	-	HAP (950 °C)	Nanosize	Plate-like	[93]
Bovine Bone	Calcination	-	HAP (750 °C)	Nanosize	Nanorod	[91]
	Calcination	1.98	HAP (850 °C)	Nanosize	-	[92]
Fish Bone	Alkaline Hydrolysis	-	HAP	<22.5 nm	Rod-like	[95]
	Calcination	1.94	HAP (900 °C)	-	Grain-Shaped	[94]
	Calcination	1.47, 1.88, 1.51	HAP (650 °C)	Nanosize	-	[98]
	Calcination	2.63, 2.14, 2.12, 2.54	HAP	0.1–4.0 μm	Irregular	[100]
Fish Scale	Precipitation	-	HAP	Nanosize	Irregular	[96]
	Calcination	1.74	HAP (900 °C)	~50 μm	Hexagonal	[99]
	Calcination	-	HAP (800°)	Nanosize	-	[97]
Eggshell	Calcination + Precipitation	1.68–1.83	HAP (900 °C)	416.9–623.6 μm	-	[101]
	Calcination + Hydrothermal	1.54	HAP	100–250 nm length	Rod-like	[102]
	Calcination + Microwave-assisted Hydrothermal	1.69	HAP	23.83 nm	Prismatic	[103]
	Calcination + Sonication	1.73	HAP	-	-	[104]
	Calcination + Ultrasonication	1.67	HAP	Nanosize	-	[105]
	UV- mediated solid state	-	HAP	-	-	[106]
	Calcination + Precipitation	1.67	HAP	100 nm	Spherical	[107]
	Calcination + Precipitation	1.67	HAP	20–50 nm	-	[108]
Oystel Shell	Microwave Irradiation	1.39–1.58	HAP	-	Rod-like	[109]
Abalone Mussel Shell	Calcination + Precipitation	1.67	HAP (1000°)	<100 μm	Agglomerate	[110]
Seashell	Precipitation	-	HAP	50–350 μm	-	[111]
Cockle Shell	Calcination + Precipitation	-	HAP (900 °C)	-	-	[112]
Snail Shell	Calcination + Precipitation	1.19	HAP, β-TCP	13.3, 15.2 nm	-	[113]

**Figure 4.** Biomedical applications of hydroxyapatite.

Another work, scrutinized the potential use of hydroxyapatite from bovine bone for dental implants and hard tissues replacement. Hydroxyapatite powder was heated at 10 °C/min until 750 °C for a 6 h dwelling time. Odusote and colleagues found that hydroxyapatite from bovine bone could be extracted using calcination and it showed excellent

**Figure 5.** Hydroxyapatite after calcination at (a) 650 °C for 6 h (b) 950 °C for another 6 h [93].

stability as it was not absorbed in the simulated body fluid; thus, it could be used for orthopedic applications. The FTIR results confirmed the characteristics and existence of phosphate, hydroxyl, and carbonate [91]. Further research revealed that hydroxyapatite with fewer pores has a higher hardness value than hydroxyapatite with more pores. The authors concluded that bovine bone was an excellent material for dental implant and bone deformity applications.

In another study, Shemshad et al. employed the calcination method to produce hydroxyapatite from bovine bone at 850 °C for 2 h when developing nanocomposite scaffolds. A heating rate of 5 °C/min was used

Table 3. Pretreatment and calcination parameters in processing mammalian bone.

Mammalian	Pretreatment (Boiling)	Drying	Heating Rate	Temperature	Dwelling Time	References
Porcine	30 min	12 h at 80 °C	10 °C/min	800 °C & 700 °C	2 h & 3 h	[90]
Ostrich	4 h	12 h at 120 °C	5 °C/min	650 °C & 950 °C	6 h	[93]
Bovine	1 h	3 weeks	10 °C/min	750 °C	6 h	[91]
Bovine	3 h	24 h at 100 °C	5 °C/min	850 °C	2 h	[92]

until the electric furnace temperature reached 850 °C. A high-energy planetary ball mill was used at a 300 rpm milling speed to produce fine powders and, thus to improve the biological and mechanical properties of hydroxyapatite. The combined bovine bone, shrimp shell, and diopside nanoparticles showed no cytotoxicity and enhanced the mechanical strength of the bone tissue [92]. The addition of hydroxyapatite from bovine bone also contributed to the enhanced bio mineralization and bioactivity of the scaffolds.

3.1.2. Fish bone and scale

The solid waste of fish scale and bone has great potential in terms of hydroxyapatite extraction as it would turn undesired waste into useful, functional material. In general, the bone or scale was first boiled to remove unwanted flesh or debris [95, 96, 98, 99]. The material was also pretreated using acetone [94], alkali treatment [95, 97], and a combination of both [98]. The majority of works in the literature employed calcination as the extraction method [94, 97, 98, 99, 100], while others used alkaline hydrolysis [95] and precipitation [96]. Table 4 summarizes the pretreatment and calcination parameters used in processing fish bone/scale.

Wardani et al. [94] reported on synthesizing hydroxyapatite from fish bone using precipitation. In their work, CaO was produced manually by bone sieving and subjected to acidic and alkali treatment between two calcination stages. As their research revealed, skipjack tuna is a potential biomaterial source for extracting hydroxyapatite. The precipitation method used to synthesize skipjack tuna produced almost uniform grain-shaped particles, and a Ca/P ratio of 1.94, while it also exhibited the best cell viability after three days through the MTT assay. The optical density of the preosteoblast cell culture response showed that 50 µg/ml was the optimum concentration after 72 h and no significant difference was identified between the samples at 24 h and 48 h (Figure 6).

Surya et al. [95] extracted hydroxyapatite using alkaline hydrolysis by heating *Sardinella longiceps* fish bone after NaOH solution treatment, followed by 5 h stirring at 400 rpm. The concept underlying this approach mainly involved removing organic elements from the mineral matrix. The undissolved dry precipitate was then sieved to collect nano hydroxyapatite powder. The authors observed that Indian oil sardine fish bone demonstrated good potential for bone replacement due to its suitable size, morphology, functional group, viability and mineralization. The XRD results showed the low crystalline properties of hydroxyapatite with an average particle size of 19.65 nm (Figure 7).

To obtain hydroxyapatite from fish scale, an aqueous precipitation reaction can be performed by mixing calcium nitrate tetrahydrate and diammonium hydrogen phosphate solutions at ambient temperature. The pH of the obtained powders was controlled using deionized water before being dried at 500 °C for 2 h. Pon-On et al. [96] found that using hydroxyapatite from Jullien's golden carp fish scale to fabricate composite, together with poly(lactic acid) and chitosan, enhanced the cell viability and alkaline phosphate activity. They also found that the combination of mineral ion-loaded hydroxyapatite and poly(lactic acid) chitosan matrix was capable of enhancing the mechanical properties of the scaffold.

On the other hand, hydroxyapatite can also be directly isolated from fish scale via calcination without the need for milling. Deb et al. [97] washed scale using distilled water and oven-dried it for 12 h at 40 °C after deproteinization with NaOH solution. The authors used *Labeo rohita* scale, finding that synthesized hydroxyapatite showed high thermal

stability beyond 800 °C. To predict the desired calcination temperature for hydroxyapatite extraction, thermogravimetric analysis (TGA) was implemented before heat decomposition in the furnace. From the observation, no degradation was noticed beyond 800 °C signifying suitable calcination temperature (Figure 8). Observation using SEM revealed that an interconnected porous structure represented pore diameters of slightly larger than 100 µm, which would fit well with biomedical applications (Figure 9). The combination of hydroxyapatite from *Labeo rohita* scale and poly(ethylene glycol) produced a scaffold with enhanced mechanical strength.

Work by Shi et al. [98] reported, the detection of CO₃²⁻ and Mg²⁺ in hydroxyapatite derived from rainbow trout and salmon bones. They compared hydroxyapatite extracted using calcination of rainbow trout, cod and salmon bone. Rainbow trout and salmon bones revealed an advantage in terms of nanohydroxyapatite production while also containing minerals essential for cell proliferation, adhesion and tissue mineralization. It was also found that the Ca/P ratios of salmon and rainbow trout bones were 1.51 and 1.47, lower than the stoichiometric ratio (1.67). Besides, it was noticed that the alkaline phosphate activity of salmon bone was higher compared to the rainbow trout and cod bones during the 72-hours culturing period, indicating that stronger interactions occurred between the salmon hydroxyapatite material and the osteoblast.

Jaffri et al. [99] employed calcination in extracting hydroxyapatite from black tilapia fish scale. At the calcination temperature of 900 °C for 3 h, it was found that Ca/P ratio of extracted hydroxyapatite equal 1.74 (Table 5). The presence of hydroxyapatite particle in hexagonal shape was shown by SEM. In addition, XRD data shows sharp peak of hydroxyapatite pattern, indicating a good potential source of hydroxyapatite for biomedical applications.

In other work, Aydin et al. [100] used bone of meagre fish to produce hydroxyapatite from natural source. The fish bone was calcined at temperature between 800 °C and 1100 °C. TGA was implemented over a temperature range of 20–1100 °C, resulting in total mass loss of approximately 59 %. They found that all calcined fish bones show hydroxyapatite and whitlockite patterns through XRD. Further, extracted hydroxyapatite demonstrate non-toxic property at concentration up to 100 mg/ml using XTT assay.

3.1.3. Shells

Starting in 2017, most literature in the Scopus database presented eggshell as a hydroxyapatite resource. Several studies used high-temperature burning calcination to produce calcium oxide, CaO [101, 104, 105, 106, 108]. The CaO was further treated with diammonium hydrogen phosphate to produce pure calcium phosphate [101, 105, 108], while other researchers used sonication with phosphoric acid [104, 107]. Other than calcination alone, the hydrothermal method [102] was also used to produce calcium chloride solution as a calcium precursor. Using the hybrid method of calcination and microwave-assisted hydrothermal [103] proved that the extraction time could be shortened. Besides, marine sources rich in calcium carbonate, CaCO₃ are oyster shell [109], abalone mussel shell [110], seashell [111], and cockle shell [112]. Calcination temperatures of 900 °C [112] and 1000 °C [110] were used to extract hydroxyapatite.

Lala et al. [101] employed calcination and disodium hydrogen phosphate to extract hydroxyapatite from eggshell. They investigated

Table 4. Pretreatment and calcination parameters in processing fish bone/scale.

Fish Bone/Scale	Pretreatment (Boiling)	Drying	Heating Rate	Temperature	Dwelling Time	References
Skipjack Tuna Bone	-	-	-	900 °C	5 h	[94]
Rohu Scale	-	12 h at 40 °C	-	1000 °C	3 h	[97]
Rainbow Trout, Cod, Salmon Bone	1 h	6 h at 60 °C	5 °C/min	650 °C	5 h	[98]
Black Tilapia Scale	1 h	3 h at 95 °C	10 °C/min	900 °C	3 h	[99]
Meagre Bone	-	2 h at 110 °C	10 °C/min	800, 900, 1000, 1100 °C	1 h	[100]

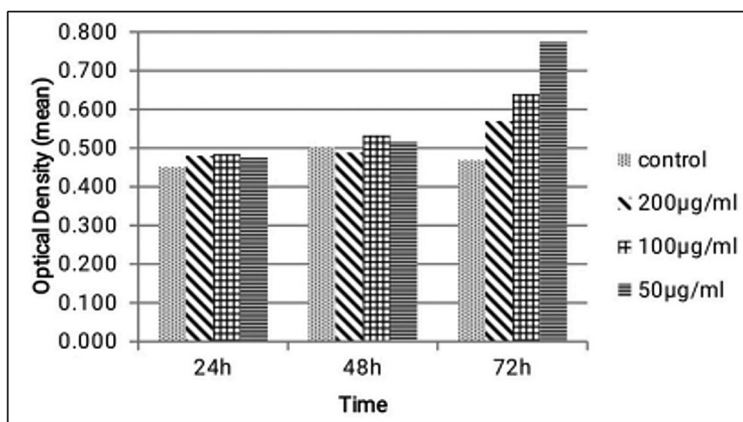


Figure 6. Viability assay of preosteoblast cell culture MC3T3-E1 [94].

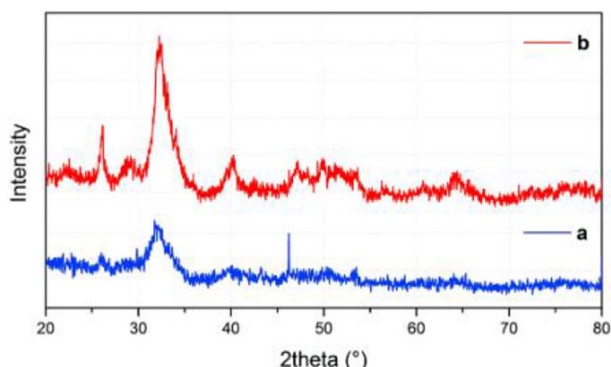


Figure 7. XRD of (a) raw bone and (b) extracted hydroxyapatite. Reproduced from Ref. [95] by permission of Elsevier.

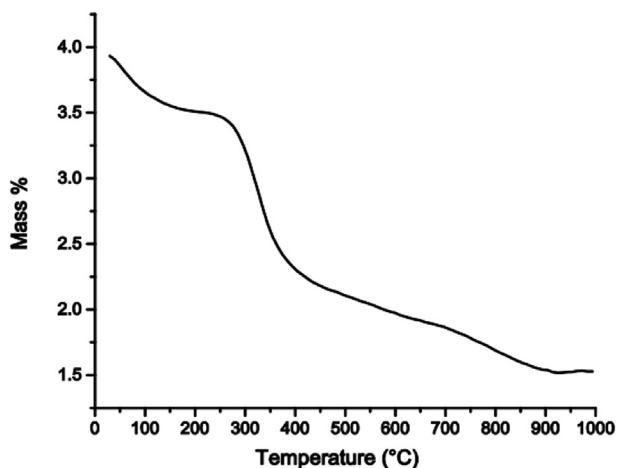


Figure 8. TGA of uncalcined fish scale [97].

the effect of aging time on the hydroxyapatite properties, concluding that 12 h aged hydroxyapatite exhibited better properties than 24 h, 36 h, and 48 h aged hydroxyapatite in terms of degradation and bioactivity. A 12 h aging time also produced a Ca/p ratio of 1.68, very close to the Ca/P ratio of human cortical bone. Previous studies involving aging time were also performed using cockle shell [114] and goniopora coral [115], whereby a 5 h aging time produced better crystallinity than a 3 h aging time for cockle shell. A higher weight percentage of hydroxyapatite was obtained for a 24 h aging time compared to a 12 h aging time for goniopora coral.

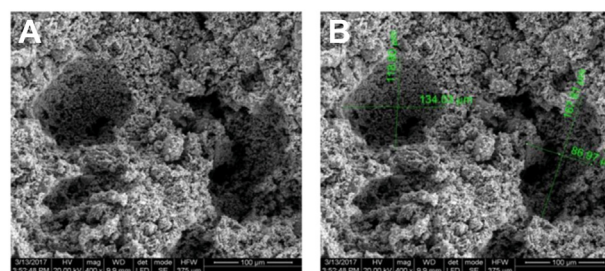


Figure 9. (a) Porous scaffold and (b) pore diameter [97].

Table 5. Ca/P molar ratio of raw and calcined black tilapia fish scale [99].

Temperature	Elemental		Average
	Ca	P	Ca/P
Raw Scale	47.68	42.63	1.11
900 °C	62.51	35.75	1.74

Nga et al. [102] successfully synthesized hydroxyapatite nanoparticles using eggshell waste as a bio-calcium precursor. In this research, the eggshell powder was initially dissolved in HCl to allow the conversion of CaCO_3 into CaCl_2 . Calcium chloride solution and Na_2HPO_4 were used as the calcium and phosphate precursors, respectively, aided by cetyl-trimethylammonium bromide through the hydrothermal method. The authors found that hydroxyapatite synthesized from eggshell enhanced the double-layer apatite formation in simulated body fluid (Figure 10). A protein adsorption test also revealed that the extracted hydroxyapatite nanoparticles displayed high protein adsorption properties after a day of incubation in a minimum essential medium (MEM).

Research conducted by Dumitrescu et al. [103], demonstrated that hydroxyapatite powder from eggshell could be prepared using the microwave-assisted hydrothermal technique. In their work, the compositional and structural similarities and differences of hydroxyapatite powder from eggshell were compared with those of partially deproteinized porcine bone (Gen-Os[®]) and totally deproteinized cortical bovine bone (Bio-Oss[®]). It was found that the eggshell sample had very high meso-porosity likely due to improved biomolecule adhesion and osteo-conductivity. The hybrid processing technique in this research also proved to be extremely fast in producing hydroxyapatite, which only took a few minutes.

Patel et al. [104] found that combining the sonication process and calcination could produce highly crystalline hydroxyapatite from eggshell, suitable for tissue engineering. The higher calcium deposition in the presence of hydroxyapatite from human mesenchymal cells

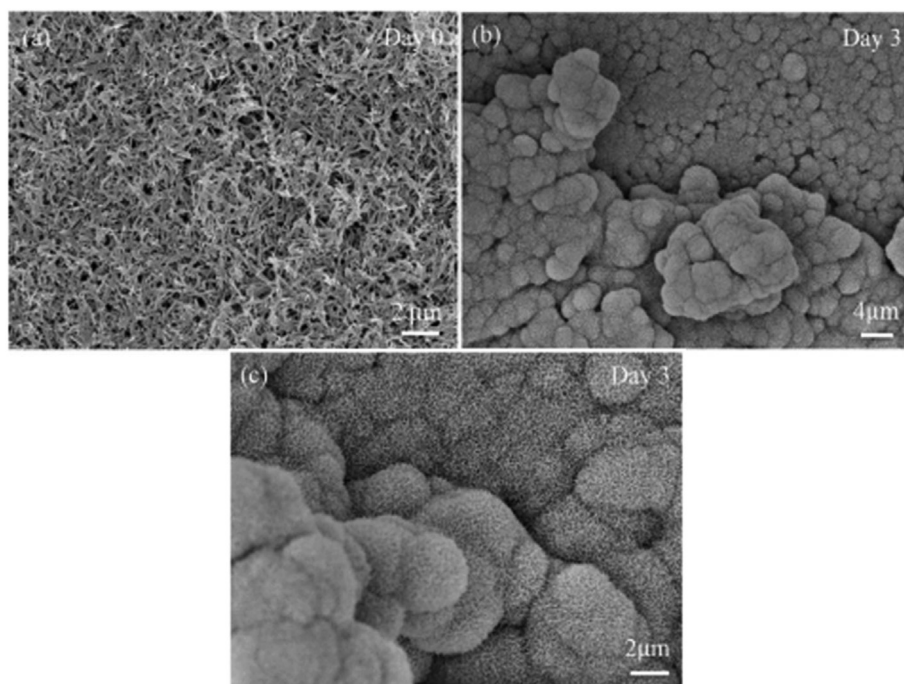


Figure 10. SEM micrographs (a) before immersion in SBF (b) after three days of immersion in SBF with 2k magnification (c) after three days of immersion in SBF with 5k magnification. Reproduced from Ref. [102] by permission of Elsevier.

indicated good osteogenic potential. Crystalline hydroxyapatite also improved cell viability, suggesting greater biocompatibility than the control sample.

The effect of hydroxyapatite from an eggshell-derived scaffold in combination with human hair keratin and jellyfish collagen was scrutinized by Arslan et al [105]. Calcium oxide from eggshell was produced by calcination in a box furnace at 900 °C for 2 h. Diammonium hydrogen phosphate was then added to produce hydroxyapatite. The researchers suggested that osteoconductive scaffold using human hair keratin, jellyfish collagen and eggshell-derived nanohydroxyapatite was a new cost-effective approach for scaffold fabrication, considering the novel and extraordinary approach of using bioceramics or biopolymers in regenerative medicine.

Sultana and colleagues [106] synthesized hydroxyapatite from eggshell without thermal treatment using a novel UV-mediated solid-state method. They suggested that hydroxyapatite can be developed using the UV-irradiation technique at room temperature, preceded by ball milling. They also observed that no significant cytotoxicity was shown from the cell viability assay. In a simulated body fluid soaking test, cell bioactivity was within the admissible range.

The research by Wu et al., found that synthesized hydroxyapatite powder from oyster shell contains magnesium and strontium [109]. The sample was prepared by mixing oyster shell powder and dicalcium phosphate dihydrate through planetary ball milling followed by sintering. It was also revealed by XRD analysis that synthesized hydroxyapatite exhibited high phase purity and good crystallinity. They also found secondary phase β -TCP in 1 and 5 h milled samples, while only primary phase was present in a 10 h milled sample.

Abalone mussel shell, which contains prismatic calcite and aragonite sheet [116], was synthesized using the precipitation method. The sample was first crushed using ball milling, followed by calcination at 1000 °C for 6 h to produce calcium oxide powder. Diammonium hydrogen phosphate was then added to the calcium oxide and distilled water mixture, which was then stirred at 70 °C for 1 h at 300 rpm velocity [110]. The authors' FTIR analysis demonstrated that no chemical decomposition had occurred for the synthesized hydroxyapatite or porous hydroxyapatite-based scaffold (Figure 11).

Sponge-shaped hydroxyapatite powder from seashell (*rapana thomasiana*) with a relatively dense fibrous structure was processed using the precipitation method [111]. It was revealed that the novel in situ precipitation method produced a strong matrix structure with a fine quality of combined collagen and hydroxyapatite. Previously, Zhang and colleagues reported that conch (*Strombus gigas*) and clam (*Tridacna gigas*) could be processed through a hydrothermal reaction to produce hydroxyapatite [117]. However, it took approximately 10 days to complete the process, which was far longer.

Afriani et al. used hydroxyapatite from cockle shell and silica as filler for a composite scaffold to examine the crystallization and degradation properties [112]. A homogenous solution of calcium and phosphate was obtained by stirring for 2.5 h at 300 rpm (at ambient temperature). The sample was then sintered for 5 h at 900 °C. It was found that the silica had slowed the degradation process of the hydroxyapatite/silica composite. On the other hand, a previous study by Sarker et al. highlighted the denaturation of collagen/silica composite in SBF and connected this observation to the *in vitro* degradation behavior as a future research topic [118].

Ahmed and teammates [113] extracted HA from snail shells using calcination and precipitation methods. The size of HA powder lies between 13.3 and 15.2 nm in diameter. HA powder from snail shell demonstrated excellent antibacterial activity against *E. coli*, *S. aureus*, *B. subtilis*, *K. pneumonia*, and *C. Albicans*. In addition, snail shell is a natural, low-cost, and biocompatible source in extracting hydroxyapatite. Using EDX, the Ca/P ratio of obtained HA powder is 1.19.

As Table 6 depicts, Mg and Na are the ions most frequently found in the reported literatures. Other trace elements such as CO_3^{2-} , K and Sr also have been observed in hydroxyapatite derived from fish bones (calcination), eggshell (calcination + hydrothermal), and oyster shell (microwave irradiation), respectively. The concentration of these elements varied depending on the differences in the animals' nutrition [119, 120, 121]. The calcium phosphate ratio of the hydroxyapatite extracted from the oyster shell varied from 1.39 to 1.58 depending on the milling time. According to Table 6, the calcination + precipitation method resulted in a higher calcium phosphate ratio compared to microwave irradiation, calcination + hydrothermal, and microwave-assisted hydrothermal

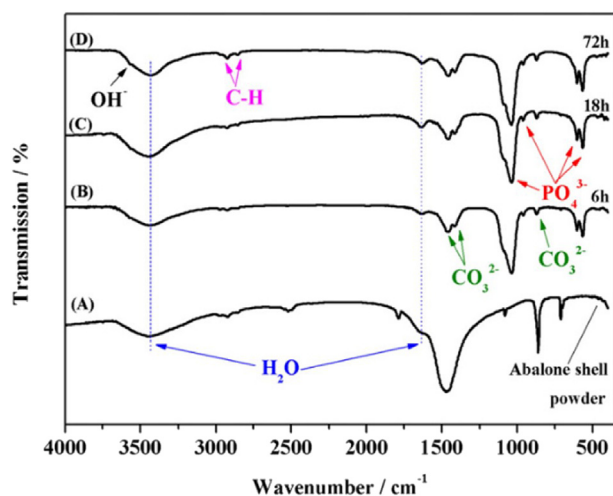


Figure 11. FTIR spectra of the raw abalone shell powders (A) and with hydrothermal times of (B) 6 h (C) 18 h and (D) 72 h. Reproduced from Ref. [116] by permission of Elsevier.

methods. Moreover, the calcination + precipitation process has produced hydroxyapatite nearer to the stoichiometric ratio (1.67) compared to the other approaches.

Hydroxyapatite from different animal sources has been extensively used in various *in vitro* evaluations due to its remarkable bioactivity, biocompatibility, and osteoconductivity. In this phase, the cell activities outside the living organism were carefully examined. Almost all the literature found in the Scopus database since 2017 (Table 7) performed *in vitro* evaluations using simulate body fluid (SBF) and/or various cell types on hydroxyapatite from porcine bone [90], bovine bone [91, 92], fish bone [94, 95, 97, 100], fish scale [96], eggshell [101, 102, 103, 104, 105, 106, 108], oyster shell [109], seashell [111], cockle shell [112], and snail shell [113]. Analysis revealed that the synthesized hydroxyapatite enhanced the apatite formation, making it similar to human bone. Rats and mice were typically used *in vitro* evaluations due to their biological and genetic comparability to humans.

4. Conclusions and future perspectives

The current review of hydroxyapatite makes several important contributions to biomedical applications. The findings attained from this review provide insights for future research. Throughout this analysis, the main keywords of “hydroxyapatite” and “biomedical applications” have been referred to review the important parameters related to hydroxyapatite extraction/synthesis. The findings to arise most clearly from this analysis are as follows:

- i. In recent years, the very readily available eggshell has been the animal source most frequently used for extracting/synthesizing natural hydroxyapatite.

Table 7. Summary of *in vitro* evaluations.

Hydroxyapatite Source	Cell/Solution	References
Porcine Bone	Rat Mesenchymal Stem Cell	[90]
Bovine Bone	Phosphate Buffered Saline	[91]
Bovine Bone	Simulated Body Fluid	[92]
Fish Bone	Preosteoblast MC3T3-E1	[94]
Fish Bone	Human Osteoblast like MG-63	[95]
Fish Scale	UMR-106	[96]
Fish Bone	Mouse Preosteoblast MC3T3-E1	[97]
Fish Bone	Human Mesenchymal Stem Cell	[100]
Eggshell	Simulated Body Fluid, Human Mesenchymal Stem Cell	[101]
Eggshell	Simulated Body Fluid, Fetal Bovine Serum	[102]
Eggshell	Amniotic Fluid Stem Cell	[103]
Eggshell	Human Mesenchymal Stem Cell	[104]
Eggshell	Human Adipose Mesenchymal Stem Cell	[105]
Eggshell	Simulated Body Fluid	[106]
Eggshell	Human Dental Pulp Stem Cell	[108]
Oyster Shell	Mouse Osteoblast MC3T3-E1	[109]
Seashell	Human Osteoblast like MG-63	[111]
Cockle Shell	Simulated Body Fluid	[112]
Snail Shell	Skin Cell HSF-4	[113]

- ii. Calcination is the preferred extraction/synthesis method, either alone or in combination with other methods, as this produces of highly crystalline hydroxyapatite powder. Faster extraction process, low-cost production, and less chemical requirement have made calcination as preferred extraction/synthesis method.
- iii. Combined methods normally employ a calcium source in the first stage, followed by mixing with phosphate source in the second stage to produce hydroxyapatite.
- iv. Trace elements such as Mg, Na, K, CO₃²⁻ and Sr were detected from hydroxyapatite synthesized using fish bones, oyster shell and eggshell. The presence of these trace elements is important in enhancing the bioactivity, differentiation, proliferation, and osteoblast activity of cells.
- v. Abalone mussel shell has exactly the right stoichiometric ratio value for hydroxyapatite powder when extracted through a combination of calcination and precipitation. Meanwhile, synthesized hydroxyapatite from eggshells has a Ca/P ratio closer to the stoichiometric ratio (1.67) than mammalian and fish bone.
- vi. *In vitro* evaluation of hydroxyapatite is commonly performed using simulated body fluid (SBF), and the cellular responses from numerous cells, for instance, human mesenchymal stem cells, rat mesenchymal stem cells, human osteoblast MG-63, and mouse osteoblast MC3T3-E1.
- vii. As a whole, it can be said that the crystallinity, density, and Ca/P ratio of calcined hydroxyapatite will increase with the increasing temperature. Besides, pH value influences the dissolution

Table 6. Presence of trace element in synthesized hydroxyapatite.

Details	Method				
	Microwave Irradiation	Calcination + Hydrothermal	Calcination + Precipitation	Calcination + Microwave-Assisted Hydrothermal	Calcination
Ca/P ratio	1.39–1.58	1.54	1.68–1.83	1.69	1.47, 1.51
Waste	Oyster Shell	Eggshell	Eggshell	Eggshell	Fish Bone
Trace Element	Mg, Sr [109]	Mg, Na, K [102]	Na [101], Na, Mg [108]	Mg, Na [103]	Mg, CO ₃ ²⁻ [98], Mg, Na [100]

behavior where ion release occurs more quickly at low pH, resulting in notably faster apatite formation.

Due to the plentiful natural resources, hydroxyapatite manufacturing will be more affordable in the near future. Early studies on the characteristics of hydroxyapatite produced from eggshell and fish bone have yielded encouraging findings [98, 100, 101, 102, 103]. The microscopic organisation and structure of a tissue made of fish bone and hydroxyapatite produced from eggshells have been studied, and the results have demonstrated similar efficacy to that of commercially available materials. Despite the fact that there are only a few comparative studies available, excellent performance has been shown. Although significant progress has been achieved in creating materials with enough mechanical strength, creating the perfect tissue engineering scaffolds using hydroxyapatite as the nano-building block still presents great obstacles. An increasing number of biomimetic structural scaffolds based on hydroxyapatite will be researched as we learn more about natural bone structure and the milieu for bone regeneration. New biomimetic or bio-inspired techniques will also be suggested for creating different hydroxyapatite-based scaffolds. By using hydroxyapatite particles as a fundamental building block, researchers can create multifunctional hydroxyapatite particles that can meet the needs of the tissue microenvironment or solve medical problems.

Declarations

Author contribution

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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