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Extraction of Natural Hydroxyapatite for Biomedical Applications – A Review

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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.

Keywords: Material science; biotechnology; health science; biomedical engineering; regenerative medicine

1. Introduction

Increasing importance is being placed on a hydroxyapatite-derived scaffold for bone tissue regeneration applications as an alternative to bone grafts [1-5]. 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 [8-11] 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 [13-15], fish bone and scale [16-18], and shells [19-21] 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 [22-25]. 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 applications [26-29]. 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 hve been published between 2012 and 2021.

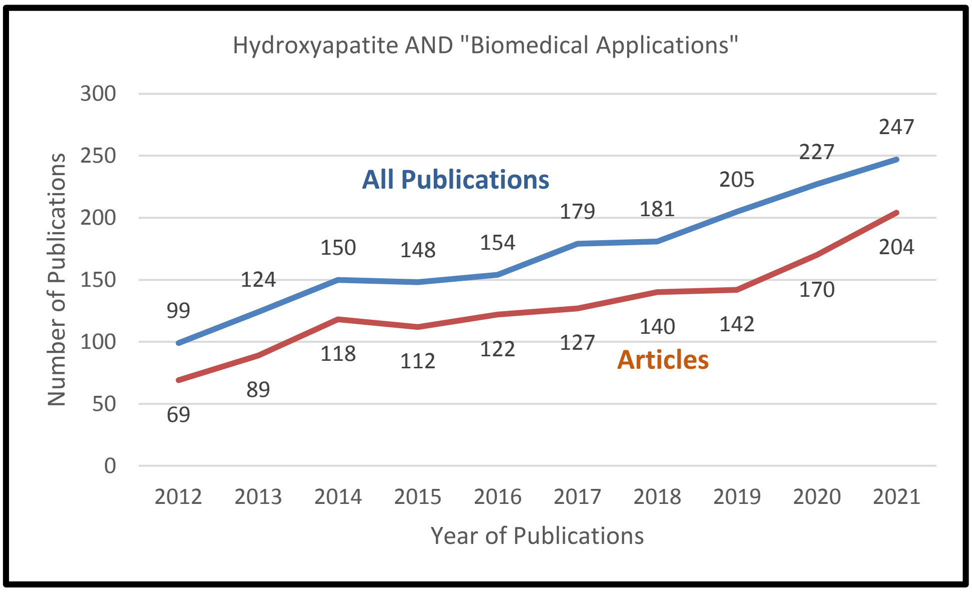

Fig.1. Published articles using keywords "hydroxyapatite" and "biomedical applications" (2012 – 2021).

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 ofthe 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 phosphatese 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 fisrt 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 [39-41], skull [42-44], and teeth [45-48]. 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, natrium, potassium, silicon, strontium, and iron [50-53]. Table 1 summarizes the biological effects of different trace elements identified in previous studies [54-79]. 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].

Tab.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]

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 [82-84]. 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.

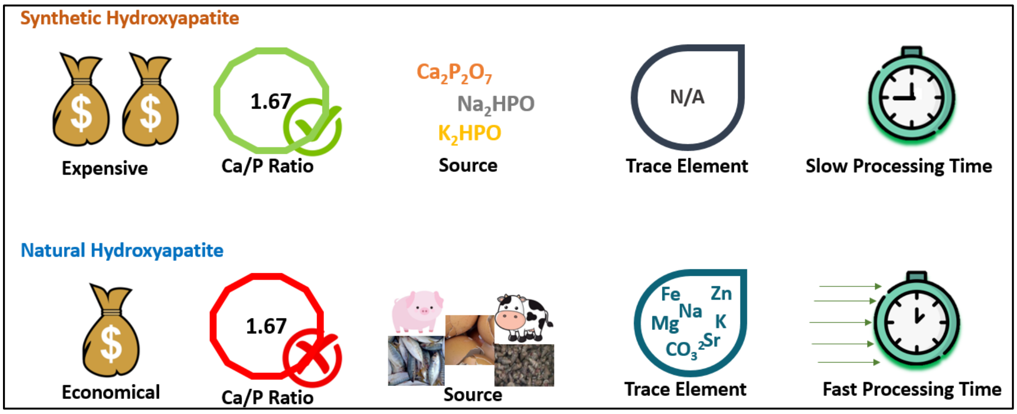

Fig.2. Natural hydroxyapatite vs synthetic hydroxyapatite.

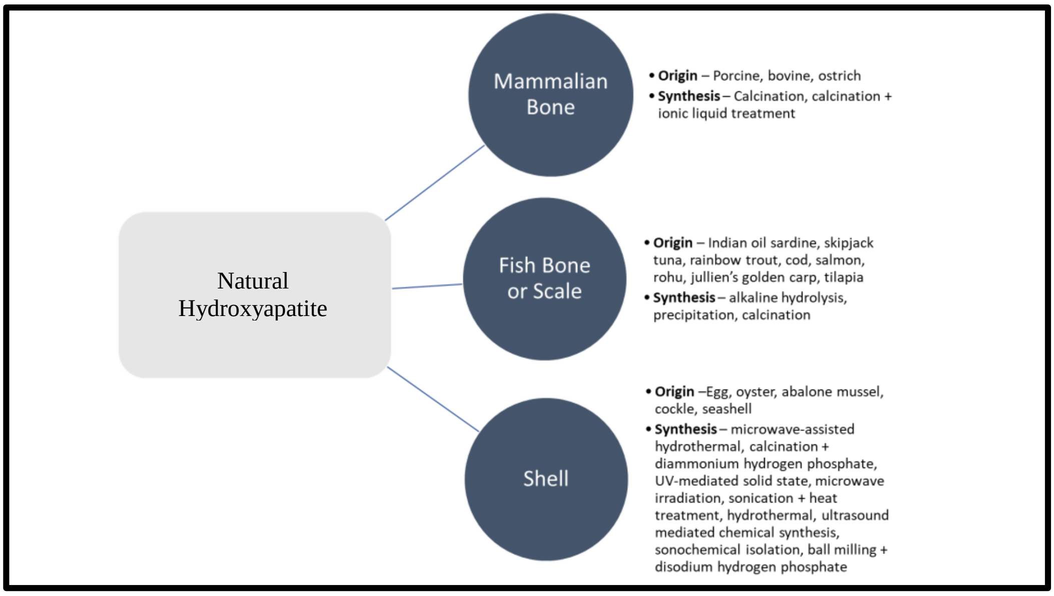

Fig.3. Origins and synthesis methodology of natural hydroxyapatite.

3.1 Biomaterials as Natural Hydroxyapatite Source

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 [86-88]. 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 promising potential for various biomedical applications owing to its biocompatible and bioactive properties. Figure 4 displays the most common biomedical applications of hydroxyapatite.

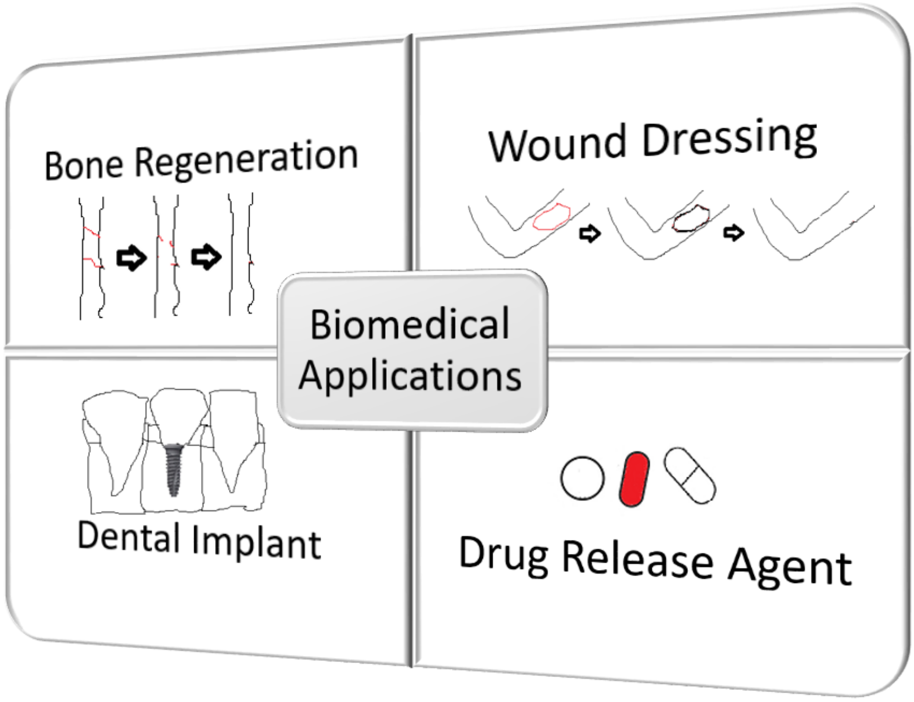

Fig.4. Biomedical applications of hydroxyapatite.

3.1.1 Mammalian Bone

Mammalian bones - for instance, porcine [89], bovine [90,91], and ostrich [92] - 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 pretreated 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 [90,92]. 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 [89]. Table 3 summarizes the pretreatment and calcination parameters used in processing mammalian bone.

Tab.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	[89]
Ostrich Bone	Calcination	-	HAP (950°C)	Nanosize	Plate-like	[92]
Bovine Bone	Calcination	-	HAP (750°C)	Nanosize	Nanorod	[90]
	Calcination	1.98	HAP (850°C)	Nanosize	-	[91]
Fish Bone	Alkaline Hydrolysis	-	HAP	<22.5 nm	Rod-like	[94]
	Calcination	1.94	HAP (900°C)	-	Grain-Shaped	[93]
	Calcination	1.47, 1.88, 1.51	HAP (650°C)	Nanosize	-	[97]
Fish Scale	Precipitation	-	HAP	Nanosize	Irregular	[95]
	Calcination	-	HAP (800°)	Nanosize	-	[96]
Eggshell	Calcination + Precipitation	1.68-1.83	HAP(900°C)	416.9-623.6 µm	-	[98]
	Calcination + Hydrothermal	1.54	HAP	100-250 nm length	Rod-like	[99]
	Calcination + Microwave-assisted Hydrothermal	1.69	HAP	23.83 nm	Prismatic	[100]
	Calcination + Sonication	1.73	HAP	-	-	[101]
	Calcination + Ultrasonication	1.67	HAP	Nanosize	-	[102]
	UV- mediated solid state	-	HAP	-	-	[103]
Oystel Shell	Microwave Irradiation	1.39-1.58	HAP	-	Rod-like	[104]
Abalone Mussel Shell	Calcination + Precipitation	1.67	HAP(1000°)	<100 µm	Agglomerate	[105]
Seashell	Precipitation		HAP	50-350 µm		[106]
Cockle Shell	Calcination + Precipitation	-	HAP(900°C)	-	-	[107]

Tab.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	[89]
Ostrich	4 h	12 h at 120 °C	5 °C/min	650 °C & 950 °C	6 h	[92]
Bovine	1 h	3 weeks	10 °C/min	750 °C	6 h	[90]
Bovine	3 h	24 h at 100 °C	5 °C/min	850 °C	2 h	[91]

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 [92]. 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 [89], 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±7.37% and 29.03±1.70%, respectively. It was also revealed through the calvarial defect implant assessment that fluorinated hydroxyapatite exhibited notably better new bone formation activities.

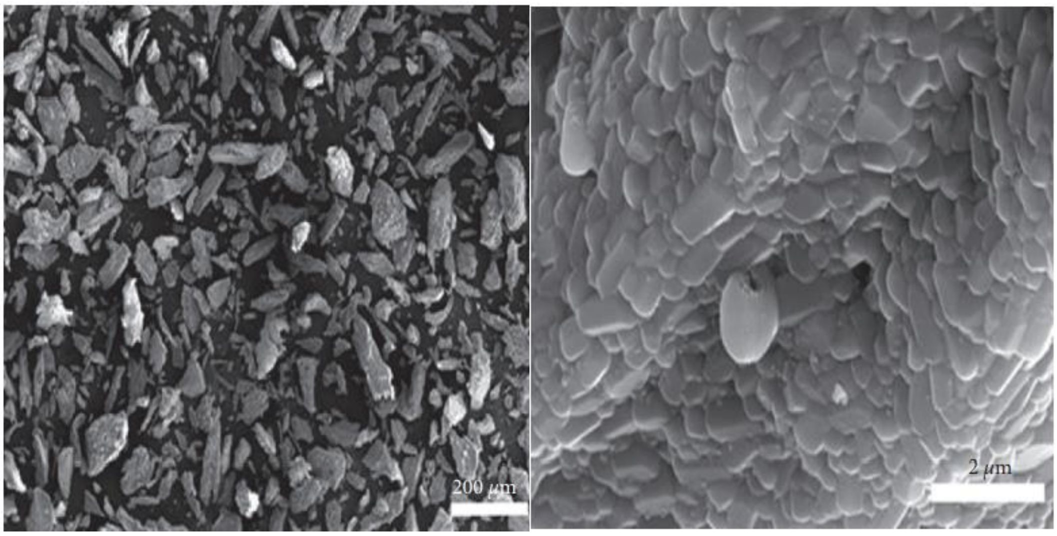

Fig.5. Hydroxyapatite after calcination at (a) 650 °C for 6 h (b) 950 °C for another 6 h [92]

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 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 [90]. 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 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 [91]. 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 [94,95,97]. The material was also pretreated using acetone [93], alkali treatment [94,96], and a combination of both [97]. The majority of works in the literature employed calcination as the extraction method [93, 96, 97], while others used alkaline hydrolysis [94] and precipitation [95]. Table 4 summarizes the pretreatment and calcination parameters used in processing fish bone / scale.

Tab.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	[93]
Rohu Scale	-	12 h at 40 °C	-	1000 °C	3 h	[96]
Rainbow Trout, Cod, Salmon Bone	1 h	6 h at 60 °C	5 °C/min	650 °C	5 h	[97]

Wardani et al. [93] 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).

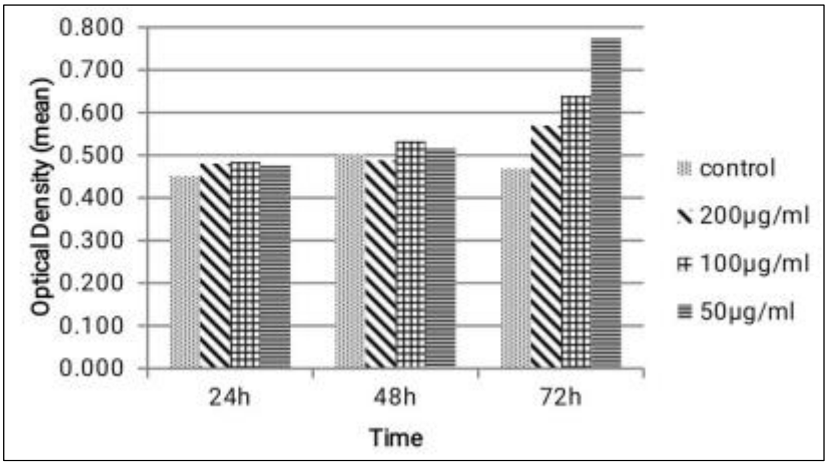

Fig.6. Viability assay of preosteoblast cell culture MC3T3-E1 [93].

Surya et al. [94] 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.

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. [95] found that using hydroxyapatite from Jullien's golden carp fish scale to fabricate composite, together with polylactic acid and chitosan, enhanced the cell viability and alkaline phosphate activity. They also found that the combination of mineral ion-loaded hydroxyapatite and polylactic 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. [96] 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, thermo gravimetric analysis (TGA) was implemented before heat decomposition in the furnace. 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 7). The combination of hydroxyapatite from *Labeo rohita* scale and polyethylene glycol produced a scaffold with enhanced mechanical strength.

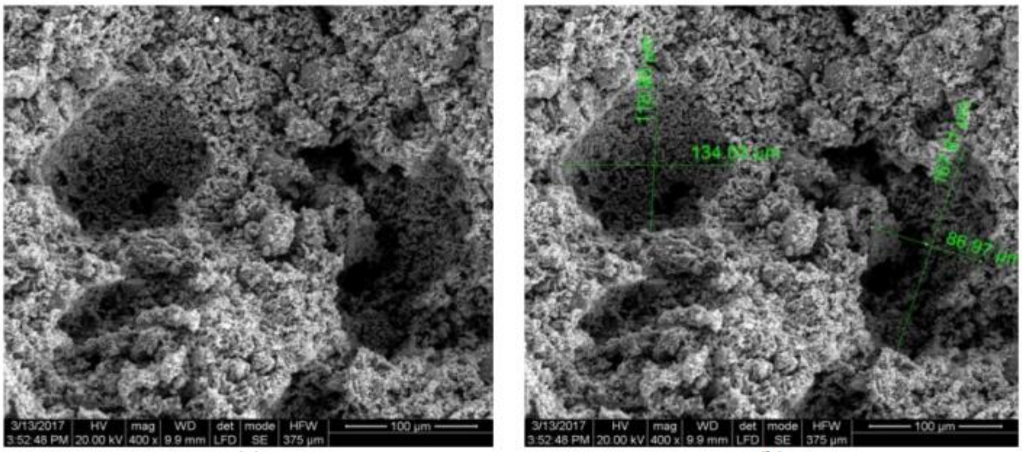

Fig.7. (a) Porous scaffold and (b) pore diameter [96].

Work by Shi et al. [97] reported, the detection of CO_3^{2} and Mg^{2+} 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.

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 [98,101,102]. The CaO was further treated with diammonium hydrogen phosphate to produce pure calcium phosphate [98,102], while other researchers used sonication with orthophosphoric acid [101]. Other than calcination alone, the hydrothermal method [99] was also used to produce calcium chloride solution as a calcium precursor. Using the hybrid method of calcination and microwave-assisted hydrothermal [100] proved that the extraction time could be shortened. Besides, marine sources rich in calcium carbonate, $CaCO_3$ are oyster shell [104], abalone mussel shell [105], seashell [106], and cockle shell [107]. Calcination temperatures of 900 °C [107] and 1000 °C [105] were used to extract hydroxyapatite.

Lala et al. [98] employed calcination and disodium hydrogen phosphate to extract hydroxyapatite from eggshell. They investigated 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 [108] and goniopora coral [109], 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.

Nga et al. [99] 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 8). 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).

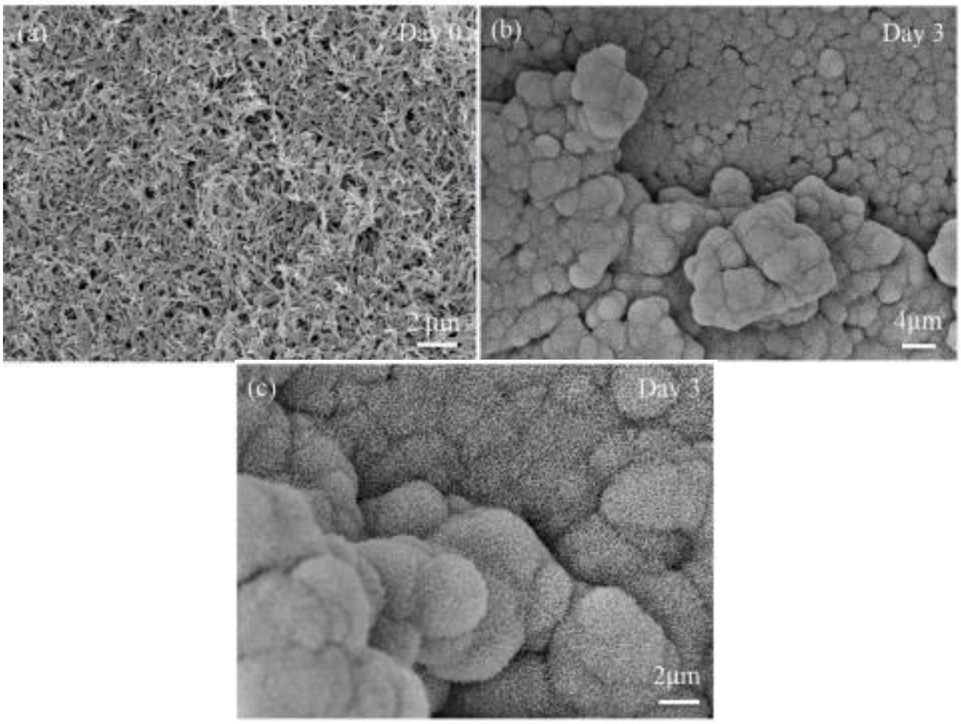

Fig.8. SEM micrographs (a) before immersion in SBF (b) after three days of immersion in SBF with 2k magnification (c) after three days of immersion in SBF with 5k magnification. Reproduced from Ref. [99] by permission of Elsevier.

Research conducted by Dumitrescu et al. [100], 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 osteoconductivity. 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. [101] 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 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 [102]. 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 collegues [103] 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 [104]. 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 [110], 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 [105]. The authors' FTIR analysis demonstrated that no chemical decomposition had occurred for the synthesized hydroxyapatite or porous hydroxyapatite-based scaffold.

Sponge-shaped hydroxyapatite powder from seashell (*rapana thomasiana*) with a relatively dense fibrous structure was processed using the precipitation method [106]. 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 [111]. 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 [107]. 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 [112].

As Table 5 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 [113-115]. 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 5, the calcination + precipitation method resulted in a higher calcium phosphate ratio compared to microwave irradiation, calcination + hydrothermal, and microwave-assisted hydrothermal methods. Moreover, the calcination + precipitation process has produced hydroxyapatite nearer to the stoichiometric ratio (1.67) compared to the other approaches.

Tab.5. 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 [104]	Mg, Na, K [99]	Na [98]	Mg, Na [100]	Mg, CO_3^2 [97]

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 6) performed in vitro evaluations using simulate body fluid (SBF) and/or various cell types on hydroxyapatite from porcine bone [89], bovine bone [90,91], fish bone [93,94,96], fish scale [95], eggshell [98-103], oyster shell [105], seashell [106], and cockle shell [107]. 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.

Tab.6. Summary of in vitro evaluations.

Hydroxyapatite Source	Cell/Solution	References
Porcine Bone	Rat Mesenchymal Stem Cell	[89]
Bovine Bone	Phosphate Buffered Saline	[90]
Bovine Bone	Simulated Body Fluid	[91]
Fish Bone	Preosteoblast MC3T3-E1	[93]
Fish Bone	Human Osteoblast like MG-63	[94]
Fish Scale	UMR-106	[95]
Fish Bone	Mouse Preosteoblast MC3T3-E1	[96]
Eggshell	Simulated Body Fluid, Human Mesenchymal Stem Cell	[98]
Eggshell	Simulated Body Fluid, Fetal Bovine Serum	[99]
Eggshell	Amniotic Fluid Stem Cell	[100]
Eggshell	Human Mesenchymal Stem Cell	[101]
Eggshell	Human Adipose Mesenchymal Stem Cell	[102]
Eggshell	Simulated Body Fluid	[103]
Oyster Shell	Mouse Osteoblast MC3T3-E1	[105]
Seashell	Human Osteoblast like MG-63	[106]
Cockle Shell	Simulated Body Fluid	[107]

4. Conclusion

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.

ii. Calcination is the preferred extraction/synthesis method, either alone or in combination with other methods, as this produces of highly crystalline hydroxyapatite powder.

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, $CO_3^{2}$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.

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Data Availability

Data sharing is not applicable to this article.

References:

[1] V.S. Kattimani, S. Kondaka, K.P. Lingamaneni, Hydroxyapatite – Past, present, and future in bone regeneration, Bone and Tissue Regeneration Insights 7 (2016) 9-19.

[2] M. Saleem, S. Rasheed, C. Yougen, Silk fibroin/hydroxyapatite scaffold: a highly compatible material for bone regeneration, Science and Technology of Advanced Materials 21(1) (2020) 242-266.

[3] J.P. Gleeson, N.A. Plunkett, F.J. O'Brien, Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration, European Cells and Materials 20 (2010**)** 218-230.

[4] G. Krishnamurthy, A review on hydroxyapatite-based scaffolds as a potential bone graft substitute for bone tissue engineering applications, Journal of the University of Malaya Medical Centre 16(2) (2013) 1-6.

[5] K.K. Tan, G.H. Tan, B.S. Shamsul, K.H. Chua, M.H. Ng, B.H. Ruszymah, B.S. Aminuddin, M.Y. Loqman, Bone graft substitute using hydroxyapatite scaffold seeded with tissue engineered autologous osteoprogenitor cells in spinal fusion: early result in

a sheep model, The Medical Journal of Malaysia 60 (2005) 53-58.

[6] A.S. Greenwald, S.D. Boden, V.M. Goldberg, Y. Khan, C.T. Laurencin, R.N. Rosier, Bone-graft substitutes: facts, fictions, and applications, The Journal of Bone and Joint Surgery 83-A (2001) 98-103.

[7] T.E. Mroz, M.J. Joyce, M.P. Steinmetz, I.H. Liebermann, J.C. Wang, Musculoskeletal allograft risks and recalls in the United States, Journal of the American Academy of Orthopaedic Surgeons 16(10) (2008) 559-565.

[8] H. Shi, Z. Zhou, W. Li, Y. Fan, Z. Li, J. Wei, Hydroxyapatite based materials for bone tissue engineering: a brief and comprehensive introduction, Crystals 11 *(*2021) 149.

[9] M. Murshed, Mechanism of bone mineralization, Cold Spring Harbour Perspective in Medicine 2018.

[10] S. Pokhrel, Hydroxyapatite: preparation, properties and its biomedical applications, Advances in Chemical Engineering and Science 8 (2018) 225-240.

[11] A. Ressler, A. Gudelj, K. Zadro, M. Antunovic, M. Cvetnic, M. Ivankovic, H. Ivankovic, From bio-waste to bone substitute: synthesis of biomimetic hydroxyapatite and its use in chitosan-based composite scaffold preparation, Chem. Biochem. Eng. 34(2) 2020 59–71.

[12] N.A.S.M. Pu'ad, P. Koshy, H.Z. Abdullah, M.I. Idris, T.C. Lee, Syntheses of hydroxyapatite from natural sources, Heliyon 5 (2019) e01588.

[13] M. Carlo, S. Antonio, P. Vittoria, I. Giovanna, P. Adriano, Maxillary sinus augmentation with a porous synthetic hydroxyapatite and bovine-derived hydroxyapatite: a comparative clinical and histologic study, International Journal of Oral & Maxillofacial Implants 22 (2007) 980-986.

[14] W. Khoo, F.M. Nor, H. Ardhyananta, D. Kurniawan, Preparation of natural hydroxyapatite from bovine femur bones using calcination at various temperatures,

Procedia Manufacturing 2 2015 196-201.

[15] A. Veremeev, R. Bolgarin, V. Nesterenko, A. Andreev-Andrievskiy, A. Kutikhin, Native bovine hydroxyapatite powder demineralised bone matrix powder, and purified bone collagen membrane are efficient in repair of critical-sized rat calvarial defects, Materials 13 (2020) 3393.

[16] B. Mondal, S. Mondal, A. Mondal, N. Mandal, Fish scale derived hydroxyapatite scaffold for bone tissue engineering, Materials Characterization 121 2016 112-124.

[17] A.N.K. Fara, H.Z. Abdullah, Characterization of natural hydroxyapatite (HAP) derived from different types of tilapia fish bones and scales, Malaysian Journal of Microscopy 10 2014 34-40.

[18] O. Gunduz, O. Kilic, N. Ekren, H. Gokce, G. Kalkandelen, F.N. Oktar, Natural hydroxyapatite synthesis from fish bones: "Atlantic bonito" (sarda sarda), Key Engineering Materials 720 2016 207-209.

[19] F. Cestari, F. Agostinacchio, A. Galotta, G. Chemello, A. Motta, V.M. Sglavo, Nano-hydroxyapatite derived from biogenic and bioinspired calcium carbonates: synthesis and in vitro bioactivity, Nanomaterials 11 2021 264.

[20] C.S. Kumar, K. Dhanaraj, R.M. Vimalathithan, P. Ilaiyaraja, G. Suresh, Hydroyapatite for bone related applications derived from sea shell waste by simpleprecipitation method, Journal of Asian Ceramic Societies 8(2) 2020 416-429.

[21] G.T. El-Bassyouni, S.S. Eldera, S.H. Kenawy, E.M.A. Hamzawy, Hydroxyapatite nanoparticles derived from mussel shells for in vitro cytoxicity test and cell viability. Heliyon 6 2020 e04085.

[22] M.S.F. Hussin, A.M. Serah, K.A. Azlan, H.Z. Abdullah, M.I. Idris, I. Ghazali, A.H.M. Shariff, N. Huda, A.A. Zakaria, A bibliometric analysis of the global trend of using alginate, gelatine, and hydroxyapatite for bone tissue regeneration applications,

Polymers 13(4) 2021 647.

[23] G.S. Krishnakumar, N. Gostynska, M. Dapporto, E. Campodoni, M. Montesi, S. Panseri, A. Tampieri, E. Kon, M. Marcacci, S. Sprio, M. Sandri, Evaluation of different crosslinking agents on hybrid biomimetic collagen-hydroxyapatite composites for regenerative medicine, International Journal of Biological Macromolecules 106 2018 739-748.

[24] R.S. Bedi, G. Chow, J. Wang, L. Zanello, Y.S. Yan, Bioactive materials for regenerative medicine: zeolite-hydroxyapatite bone mimetic coatings, Advanced Engineering Materials 14 2012 200-206.

[25] R. Zhang, N. Metoki, O. Sharabani-Yosef, H. Zhu, N. Eliaz, Hydroxyapatite/mesoporous graphene/single-walled carbon nanotubes freestanding flexible hybrid membranes for regenerative medicine, Advanced Functional Materials 2016 7965-7974.

[26] S. George, D. Mehta, V.K. Saharan, Application of hydroxyapatite and its modified forms as adsorbents for water defluoridation: an insight into process synthesis, Reviews in Chemical Engineering 36(3) 2018 369-400.

[27] J.A. Da Cruz, W.R. Weinand, A.M. Neto, R.S. Palacios, A.J.M. Sales, P.R. Prezas, M.M. Costa, M.P.F. Graca, Low-Cost hydroxyapatite powders from tilapia fish, Advanced Manufacturing for Biomaterials and Biological Materials 72 2020 1435-1442.

[28] E. Pepla, L.K. Besherat, G. Palaia, G. Tenore, G. Migliau, Nano-hydroxyapatite and its applications in preventive, restorative, and regenerative dentistry: a review of literature, Annali di Stomatologia 3 2014 108-114.

[29] M.N. Salimi, A. Anuar, Characterizations of biocompatible and bioactive hydroxyapatite particles, Procedia Engineering 53 2013 192-196.

[30] F.H. Albee, H.F. Morrison, Studies in bone growth, Annals of Surgery 71(1) 1920 32-

39.

[31] K.O. Haldeman, J.M. Moore, Influence of a local excess of calcium and phosphorus on the healing of fracture: an experimental study, Archives of Surgery 29(3) 1934 385-396.

[32] C.B. Huggins, H.R. Mccarroll, B.H. Blocksom, Experiments on the theory of osteogenesis the influence of local calcium deposits on ossification; the osteogenic stimulus of epithelium, Archives of Surgery 32 1936 915-931.

[33] S.L. Bahn, Plaster: a bone substitute, Oral Surgery, Oral Medicine, Oral Pathology 21(5) 1966 672-681.

[34] C.B. Huggins, M.R. Urist, Dentin matrix transformation: rapid induction of alkaline phosphatase and cartilage, Science 167 1970 896-898.

[35] K. Holmstrand, Biophysical investigations of bone transplants and bone implants: an experimental study, Acta Orthopaedica Scandinavica 28 1957 1-66.

[36] D.R. Robert, D. James, G. Park, M. Garth, Bone generation: an experimental study of bone-grafting materials, The Journal of Bone and Joint Surgery 34(3) 1952 638-647.

[37] E.P. Barrett, J.M. Brown, S.M. Oleck, Some granular carbonaceous adsorbents for sugar refining, Ind. Eng. Chem. 43 1951 639-654.

[38] M.F. Basle, A. Rebel, F. Grizon, G. Daculsi, N. Passuti, R. Filmon, Cellular response to calcium phosphate ceramics implanted in rabbit bone, Journal of Material Science: Materials in Medicine 4 1993 273-280.

[39] S. Von Euw, Y. Wang, G. Laurent, C. Drouet, F. Babonneau, N. Nassif, T. Azais, Bone mineral: new insights into its chemical composition, Scientific Report 9 2019 8456.

[40] J.M. Spivak, A. Hasharoni, Use of hydroxyapatite in spine surgery**,** European Spine Journal 2 2001 197-204.

[41] B.D. Hahn, D.S. Park, D.S. Choi, J.J. Ryu, W.H. Yoon, J.H. Choi, J.W. Kim, C.W. Ahn, H.E. Kim, B.H. Yoon, I.K. Jung, Osteoconductive hydroxyapatite coated PEEK for

spinal fusion surgery, Applied Surface Science 283 2013 6-11.

[42] R. Verheggen, H.A. Merten, Correction of skull defects using hydroxyapatite cement (HAC) – evidence derived from animal experiments and clinical experience, Acta Neurochirurgica 143 2001 919-926.

[43] J. Wiltfang, P. Kessler, M. Buchfelder, H.A. Merten, F.W. Neukam S. Rupprecht, Reconstruction of skull bone defects using the hydroxyapatite cement with calvarial split transplants, Journal of Oral and Maxillofacial Surgery 62 2004 29-35.

[44] J.L. Weissman, C.H. Snyderman, B.E. Hirsch, Hydroxyapatite cement to repair skull base defects: radiologic appearance, American Journal of Neuroradiology 17 1996 1569-1574.

[45] B.T. Amaechi, P.A. AbdulAzees, D.O. Alshareif, M.A. Shehata, P.P.C.S. Lima, A. Abdollahi, P.S. Kalkhorani, V. Evans, Comparative efficacy of a hydroxyapatite and a fluoride toothpaste for prevention and remineralization of dental caries in children, BDJ Open 5 2019 18.

[46] M. Memarpour, F. Shafiei, A. Rafiee, M. Soltani, M.H. Dashti, Effect of hydroxyapatite nanoparticles on enamel remineralization and estimation of fissure sealant bond strength to remineralized tooth surfaces: an in vitro study, BMC Oral Health 19 2019 92.

[47] C.M.G. Nobre, N. Putz, M. Hannig, Adhesion of hydroxyapatite nanoparticles to dental materials under oral conditions, Hindawi 2020 6065739.

[48] M. Bossu, M. Saccucci, A. Salucci, G.D. Giorgio, E. Bruni, D. Uccelletti, M.S. Sarto, G. Familiari, M. Relucenti, A. Polimeni, Enamel remineralization and repair results of biomimetic hydroxyapatite toothpaste on deciduous teeth: an efective option to fuoride toothpaste, Journal of Nanobiotechnology **2**019 17.

[49] D. Mehta, S. George, P. Mondal, Synthesis of hydroxyapatite by chemical precipitation technique and study of its biodegradability, International Journal of Research in Advent

Technology 2(4) 2014 159-161.

[50] J. Liu, R. Yao, J. Guo, T. Gao, J. He, G. Meng, F. Wu, The regulating effect of trace elements Si, Zn and Sr on mineralization of gelatin-hydroxyapatite electrospun fiber, Colloids and Surfaces B: Biointerfaces 204 2021 111822.

[51] R.M.G. Brehtana, J.R. Guerra-Lopez, L.A. de Sena, An overview on biological effects of trace-element in substituted calcium phosphates, VII Latin American Congress on Biomedical Engineering CLAIB 2017 60.

[52] I. Zofkova, P. Nemcikova, P. Matucha, Trace elements and bone health, Clinical Chemistry and Laboratory Medicine 51(8) 2013 1555-1561.

[53] Z. Qamar, H.P. Chew, T. Fatima, A.R. Zubaidah, Influence of trace elements on dental enamel properties: a review, Journal of the Pakistan Medical Association 67(1) 2017 116-120.

[54] T.J. Webster, C. Ergun, R.H. Doremus, R. Bizios, Hydroxylapatite with substituted magnesium, zinc, cadmium, and yttrium. II. mechanisms of osteoblast adhesion, Journal of Biomedical Materials Research 59 2001 312-317.

[55] D.V. Shepherd, K. Kauppinen, R.A. Brooks, S.M. Best, An in vitro study into the effect of zinc substituted hydroxyapatite on osteoclast number and activity, Journal of Biomedical Materials Research 102 2014 4136-4141.

[56] A. Ito, M. Otsuka, H. Kawamura, M. Ikeuchi, H. Ohgushi, Y. Sogo, N. Ichonose, Zinc-containing tricalcium phosphate and related materials for promoting bone formation, Current Applied Physics 5 2005 402-406.

[57] I.R. De Lima, G.G. Alves, G.V.O. Fernandes, E.P. Dias, G.A. Soares, J.M. Granjeiro, Evaluation of the in vivo biocompatibility of hydroxyapatite granules incorporated with zinc ions, Materials Research 13(4) 2010 563-568.

[58] K. Ishikawa, Y. Miyamoto, T. Yuasa, A. Ito, M. Nagayama, K. Suzuki, Fabrication of

Zn containing apatite cement and its initial evaluation using human osteoblastic cells, Biomaterials 2(2) 2002 423-428.

[59] M. Ikeuchi, A. Ito, Y. Dohi, H. Ohgushi, H. Shimaoka, K. Yonemasu, T. Tateishi, Osteogenic differentiation of cultured rat and human bone marrow cells on the surface of zinc-releasing calcium phosphate ceramics, Journal of Biomedical Materials Research Part A 67A 2003 1115-1122.

[60] P. Bhattacharjee, H. Begam, A. Chanda, S.K. Nandi, Animal trial on zinc doped hydroxyapatite: a case study, Journal of Asian Ceramic Societies 2 2014 44-51.

[61] W. Yu, T.W. Sun, C. Qi, Z. Ding, H. Zhao, S. Zhao, Z. Shi, Y.J. Zhu, D. Chen, Y. He, Evaluation of zinc-doped mesoporous hydroxyapatite microspheres for the construction of a novel biomimetic scaffold optimized for bone augmentation, International Journal of Nanomedicine 12 2017 2293-2306.

[62] R.C. Cuozzo, S.C. Sartoretto, R.F.B. Resende, A.T.N.N. Alves, E. Mavropoulus, M.H.P. da Silva, M.D.C., Maia, Biological evaluation of zinc-containing calcium alginate-hydroxyapatite composite microspheres for bone regeneration, Journal of Biomedical Materials Research Part B: Applied Biomaterials 108 2020 2610-2620.

[63] K. Matsunaga, First-principles study of substitutional magnesium and zinc in hydroxyapatite and octacalcium phosphate, The Journal of Chemical Physics 128 2008 245101.

[64] C.Yanli, Simultaneous incorporation of magnesium and flurione ions in hydroxyapatite coatings on Ti6Al4V implant, PhD. Nanyang Technology University, Singapore. 2011.

[65] L. Kavitha, B. Gopi, S. Ramya, Synthesis, characterization and In vitro studies of magnesium, fluoride co-substituted hydroxyapatite nanoparticles for dental applications, 3rd Euro Congress and Expo on Dental & Oral Health 14(3) 2015.

[66] K.T. Arul, J.R. Ramya, Multifunctional behaviour of magnesium/calcium phosphate,

International Journal of Engineering Sciences & Research Technology 6(8) 2017 15-18.

[67] L.M. Da Silva, D.S. Tavares, E.A. dos Santos, Isolating the effects of Mg^{2+}, Mn^{2+}, and Sr^{2+} ions on osteoblast behavior from those caused by hydroxyapatite transformation, Materials Research 23(2) 2020 e20200083.

[68] Z. Li, B. Huang, S. Mai, X. Wu, H. Zhang, W. Qiao, X. Luo, Z. Chen, Effects of fluoridation of porcine hydroxyapatite on osteoblastic activity of human MG63 cells, Science and Technology of Advanced Materials 15 2015 035006.

[69] T. Aoba, The Effect of fluoride on apatite structure and growth, Critical Reviews in Oral Biology & Medicine 8(2) 1997 136-153.

[70] S. Liu, H. Zhou, H. Liu, H. Ji, W. Fei, E. Luo, Fluorine-contained hydroxyapatite suppresses bone resorption through inhibiting osteoclasts differentiation and function in vitro and in vivo, Cell Proliferation 52 2019 e12613.

[71] A.K. Swadi, Z.W. Jassim, M.U. Kadum M.R.M. Ali, Influence of fluoride addition on hydroxyapatite prepared for medical applications, Baghdad Science Journal 9(3) 2012 541-547.

[72] J. Gao, M. Wang, C. Shi, L. Wang, D. Wang, Y. Zhu, Synthesis of trace element Si and Sr codoping hydroxyapatite with non-cytotoxicity and enhanced cell proliferation and differentiation, Biological Trace Element Research 174 2016 208-217.

[73] T. Sun, M. Wang, Y. Shao, L. Wang, Y. Zhu, The effect and osteoblast signaling response of trace silicon doping hydroxyapatite, Biological Trace Element Research 181 2018 82-94.

[74] A.I. Rodrigues, R.L. Reus, C.A. Blitterswijk, I.B. Leonor, P. Habibovic, Calcium phosphates and silicon: exploring methods of incorporation, Biomaterials Research 2017 6.

[75] W. Yao, W.X. Xing, W. Dalin, Silicon-doped nano-hydroxyapatite: solubility, anti-

fracture and compressive strengths, Chinese Journal of Tissue Engineering Research 20 2016 435-440.

[76] Y. Yamada, T. Inui, Y. Kinoshita, Y. Shigemitsu, M. Honda, K. Nakano, H. Matsunari, M. Nagaya, H. Nagashima, M. Aizawa, Silicon-containing apatite fiber scaffolds with enhanced mechanical property express osteoinductivity and high osteoconductivity, Journal of Asian Ceramic Societies 7(2) 2019 101-108.

[77] C. Ehret, R. Aid-Launais, T. Sagardoy, R. Siadous, R. Bareille, S. Rey, S. Pechev, L. Etienne, J. Kalisky, E. de Mones, D. Letourneur, J.A. Vilamitjana, Strontium-doped hydroxyapatite polysaccharide materials effect on ectopic bone formation, PLoS One 12(9) 2017 e0184663.

[78] F. Olivier, N. Rochet, S. Delpeux-Ouldriane, J. Chancolon, V. Sarou-Kanian, F. Fayon, S. Bonnamy, Strontium incorporation into biomimetic carbonated calcium-deficient hydroxyapatite coated carbon cloth: biocompatibility with human primary osteoblasts, Mater Sci Eng C Mater Biol Appl. 116 2020 111192.

[79] M. Ge, K. Ge, F. Gao, W. Yan, H. Liu, L. Xue, H. Ma, J. Zhang, Biomimetic mineralized strontium-doped hydroxyapatite on porous poly(l-lactic acid) scaffolds for bone defect repair, International Journal of Nanomedicine 13 2018 1707-1721.

[80] A. Oryan, S. Alidadi, A, Moshiri, Current concerns regarding healing of bone defects, Hard Tissue 2(2) 2013 13.

[81] K.S. Vecchio, X. Zhang, J.B. Massie, M. Wang, C.W. Kim, Conversion of bulk seashells to biocompatible hydroxyapatite for bone implants, Acta Biomaterialia 3(6) 2007 910-918.

[82] Y.C. Huang, P.C. Hsiao, H.J. Chai, Hydroxyapatite extracted from fish scale: effects on MG63oesteoblasts-like cells, Ceramics International 37(6) 2011 1825-1831.

[83] C.M. De Assis, L.C.O. Vercik, M.L. dos Santos, M.V.L. Fook, A.C. Guastaldi,

Comparison of crystallinity between natural hydroxyapatite and synthetic cp-Ti /HA coatings, Materials Research 8 2005 207-211.

[84] M.K. Herliasyah, D.A. Nasution, M. Hamdi, A. Ide-Ektessabi, M.W. Wildan, A.E. Tontowi, Preparation and characterization of natural hydroxyapatite: a comparative study of bovine bone hydroxyapatite and hydroxyapatite from calcite, Materials Science Forum 561 2007 1441-1444.

[85] S. Sathiyavimal, S. Vasantharaj, F. LewisOscar, R. Selvaraj, K. Brindhadevi, A. Pugazendhi, Natural organic and inorganic-hydroxyapatite biopolymer composite for biomedical applications, Progress in Organic Coatings 147 2020 105858.

[86] D.S. Gomes, A.M. Santos, G.A. Neves, R.R. Menezes, A brief review on hydroxyapatite production and use in biomedicine, Ceramica 65 2019 374.

[87] M. Mucalo, Hydroxyapatite (HAp) for Biomedical Applications, Woodhead Publishing Series in Biomaterials, 2015.

[88] H. Yoshikawa, A. Myoui, Bone tissue engineering with porous hydroxyapatite ceramics, Journal of Artificial Organs 8 2005 131-136.

[89] R. Liu, W. Qiao, B. Huang, Z. Chen, J. Fang, Z. Li, Z. Chen, Fluorination enhances the osteogenic capacity of porcine hydroxyapatite, Tissue Engineering: Part A 24 2018 15-16.

[90] J.K. Odusote, Y. Danyuo, A.D. Baruwa, A.A. Azeez, Synthesis and characterization of hydroxyapatite from bovine bone for production of dental implants, Journal of Applied Biomaterials & Functional Materials 17(2) 2019 228080001983682.

[91] S. Shemshad, S. Kamali, A. Khavandi, S. Azari, Synthesis, characterization and in-vitro behavior of natural chitosan-hydroxyapatite-diopside nanocomposite scaffold for bone tissue engineering, International Journal of Polymeric Materials and Polymeric Biomaterials 68(9) 2018 516-526.

[92] K.P. Malla, S. Regmi, A. Nepal, S. Bhattarai, R.J. Yadav, S. Sakurai, R. Adhikari, Extraction and characterization of novel natural hydroxyapatite bioceramic by thermal decomposition of waste ostrich bone, International Journal of Biomaterials 2020 1690178.

[93] S.C. Wardani, H. Sujuti, E. Mustamsir, D.N. Hapsari, Synthesis and potential of skipjack tuna bone hydroxyapatite as bone tissue engineering biomaterial, Journal of Physics: Conference Series 1665 2020 012032.

[94] P. Surya, A. Nithin, A. Sundaramanickam, M. Sathish, Synthesis and characterization of nano-hydroxyapatite from sardinella longiceps fish bone and its effects on human osteoblast bone cells, Journal of Mechanical Behavior of Biomedical Materials 119 2021 104501.

[95] W. Pon-On, P. Suntornsaratoon, N. Charoenphandhu, J. Thongbunchoo, N. Krishnamra, I.M. Tang, Synthesis and investigations if mineral ions-loaded apatite from fish scale and PLA/chitosan composite for bone scaffold, Materials Letters 221 2018 143-146.

[96] P. Deb, A.B. Deoghare, E. Barun, Polyethylene glycol/fish scale-derived hydroxyapatite composite porous scaffold for bone tissue engineering, IOP Conf. Series: Materials Science and Engineering 377 2018 012009.

[97] P. Shi, M. Liu, F. Fan, C. Yu, W. Lu, M. Du, Characterization of natural hydroxyapatite originated from fish bone and its biocompatibility with osteoblasts, Materials Science & Engineering C 90 2018 706-712.

[98] S.D. Lala, E. Barua, P. Deb, A.B. Deoghare, Physico-chemical and biological behavior of eggshell bio-waste derived nano-hydroxyapatite matured at different aging time, Materials Today Communications 27 2021 102443.

[99] N.K. Nga, N.T.T. Chau, P.H. Viet, Facile synthesis of hydroxyapatite nanoparticles mimicking biological apatite from eggshells for bone-tissue engineering, Colloids and

Surfaces B: Biointerfaces 172 2018 769-778.

[100] C.R. Dumitrescu, I.A. Neascu, V.A. Surdu, A.I. Nicoara, F. Iordache, R. Trusca, L.T. Ciocan, A. Ficai, E. Andronescu, Nano-hydroxyapatite vs. xenografts: synthesis, characterization, and in vitro behavior, Nanomaterials 11 2021 2289.

[101] D.K. Patel, B. Jin, S.D. Dutta, K. Lim, Osteogenic potential of human mesenchymal stem cells on eggshells-derived hydroxyapatite nanoparticles for tissue engineering, Journal of Biomedical Material Research B Applied Biomaterials 108(5) 2019 1953-1960 .

[102] Y.E. Arslan, T.S. Arslan, B. Derkus, E. Emregul, K.C. Emregul, Fabrication of human hair keratin/jellyfish collagen/eggshell-derived hydroxyapatite osteoinductive biocomposite scaffolds for bone tissue engineering: from waste to regenerative medicine products, Colloids and Surfaces B: Biointerfaces 154 2017 160-170.

[103] S. Sultana, M.S. Hossain, M. Mahmud, M. Mobarak, M.D. Kabir, N. Sharmin, S. Ahmed, UV-assisted synthesis of hydroxyapatite from eggshells at ambient temperature: cytotoxicity, drug delivery and bioactivity, RSC Advances 11 2021 3686.

[104] S.C. Wu, Y. Kao, Y. Lu, H. Hsu, W. Ho, Precipitation and characterization of microrod hydroxyapatite bundles obtained from oyster shells through microwave irradiation, Journal of Australian Ceramic Society 2021.

[105] M. Sari, P. Hening, I.D. Chotimah, Ana, Y. Yusuf, Bioceramic hydroxyapatite-based scaffold with a porous structure using honeycomb as a natural polymeric porogen for bone tissue engineering, *B*iomaterials Research 25(2) 2021.

[106] G.T. Tihan, V. Sereanu, A. Meghea, G. Voicu, M.G. Albu, V. Mitran, A. Cimpean, R.G. Zgarian, Innovative methodology for developing a bone grafting composite biomaterial starting from the seashell of Rapana thomasiana, Comptes Rendus Chimie 20 2017 440-445.

[107] F. Afriani, Y. Tiandho, J. Evi, A. Indriawati, R.A. Rafsanjani, Synthesis and characterization of hydroxyapatite/silica composites based on cockle shells waste and tin tailings, IOP Conf. Series: Earth and Environmental Science 353 2019 012032.

[108] S.H. Saharudin, J.H. Shariffuddin, N.I.A.A. Nordin, A. Ismail, Effect of aging time in the synthesis of biogenic hydroxyapatite derived from cockle shell, Materials Today: Proceedings 19 2019 1208–1215.

[109] M.S. Mehta, R.P. Singh, Effects of aging time and sintering temperatures on thermal, structural and morphological properties of coralline hydroxyapatite, Journal of Nuclear Physics, Material Sciences, Radiation and Applications 3 2016 223–237.

[110] J. Chen, Z. Wen, S. Zhong, Z. Wang, J. Wu, Q. Zhang, Synthesis of hydroxyapatite nanorods from abalone shells viahydrothermal solid-state conversion, Materials and Design 87 2015 445-449.

[111] X. Zhang, K.S. Vecchio, Creation of dense hydroxyapatite (synthetic bone) by hydrothermal conversion of seashells, Materials Science and Engineering: C 26 2006 1445-1450.

[112] B. Sarker, S. Lyer, A. Arkudas, A.R. Boccaccini, Collagen/silica nanocomposites and hybrids for bone tissue engineering, Nanotechnology Reviews 2(4) 2013 427-447.

[113] M. Lopez-Alonso, Trace minerals and livestock: not too much not too little, ISRN Veterinary Science 2012 704825.

[114] L. Tuhy, A. Dmytryk, M. Samoraj, K. Chojnacka, Trace elements in animal nutrition. Chapter 16, Recent Advances in Trace Elements 2018.

[115] W. Godden, “Trace” elements in human and animal nutrition, Journal of the Society of Chemical Industry 58(34) 2007 791-796.