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Processing and Characterization of Cockle Shell Calcium Carbonate ($CaCO_3$) Bioceramic for Potential Application in Bone Tissue Engineering

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Abstract

Cockle shell $CaCO_3$ bioceramic is potential for multiple tissue engineering applications. The powder was produced by cleansing the cockle shells to remove all the dirt from the shell's surface followed by crushing them into $CaCO_3$ powder. The powder was characterized using X–Ray Diffraction (XRD), Scanning Electron Microscope (SEM), Energy Dispersive X-Ray Analyser (EDXA) and Fourier Transform Infrared (FT-IR). Aragonite phase was observed in cockle shell $CaCO_3$ powder and calcite phase was observed in commercial $CaCO_3$ by XRD. SEM analysis revealed the structure of cockle shell $CaCO_3$ powder to be rod-like aragonite crystals whereas commercial $CaCO_3$ had cube-like calcite crystals. The EDX outcomes showed that the cockle shell $CaCO_3$ powder had more carbon and oxygen as compared to commercial $CaCO_3$. FT-IR results also attested aragonite phase in cockle shell $CaCO_3$ powder and it also showed the existence of carbonate groups in cockle shell $CaCO_3$ powder as well as commercial $CaCO_3$ powder.

Keywords: Bioceramics; $CaCo_3$; Bone tissue engineering

Introduction

Tissue engineering is not only the formation of implants used to replace organs and tissues but it is also the manufacturing and synthesis of active biological materials that help in the repair of damaged tissues and conservation of important functions of living organisms. It is a discipline that combines together the technology, engineering materials, knowledge of cells, and appropriate biochemical elements to create synthetic tissues and organs, or to restore injured tissues. The process comprises of sowing of cells on a scaffold and then finally inserting them into the body when matured. After the scaffold is inserted into the body, the natural process of tissue regeneration occurs, the blood vessels penetrates the structure and the scaffold is eventually degraded leading to a newly formed tissue in place [1]. The Centers for Disease Control (CDC) reported that around 40 million people in the United States of America were aged 65 and above, accounting for 13% of the total population. In 2030, this percentage is expected to rise up to twice as large as in the year 2000, growing from 35 million to 72 million representing 20% of the total population. This growth in aging people is expected to push the growth of ophthalmic, reconstructive joint replacement, dental and neuromodulation implants markets [2].

Changes in material's design have always helped in the development of bone tissue engineering. Several reasons resulted in the development of bone tissue-engineered alternatives. The natural sources of biomaterials have been very limited to porcine and bovine bones, human bone itself and corals. Some of them had environmental issues whereas porcine bones are classified as 'non halal' for Muslims [3]. Biomaterials have been improving the quality of lives for many people over the past years. They have vast number of applications such as artificial skin and arteries, limb and joint replacements etc. These materials are mainly used for replacing unhealthy tissues to increase life expectancy of individuals. Scientists are trying to produce improved and new implant materials to meet the increasing demand. They are extensively studied in tissue engineering researches. Bio-resorb able and biodegradable materials are clinically and experimentally studied to design scaffolds with particular characteristics.

Cockle (a common name for a group of (mostly) small, edible, saltwater clams, marine bivalve molluscs in the family Cardiidae) shell, also known as '*Anadara granosa*' is one of the most common sources of calcium carbonate found in Malaysia which can easily fulfill the increasing demand of biomaterials due to their low cost and availability. Calcium is one of the most important elements for an organism, as it controls the passage of organic substances and inorganic ions through the cell membranes in a metabolic process also involving the removal and distribution of reaction products from the cell [4]. It is a building material for bone tissues, its inorganic part. Solid residuals of the bone tissues contain 70% of calcium hydroxyapatite and 30% of collagen fiber [4].

$CaCO_3$ is found all over the world and covers more than four percent (4%) of the total earth's crust. It is found in many common forms as limestone, chalk and marble that is mainly produced from the shells of snails, corals and shell fishes. $CaCO_3$ is one of the most versatile materials that have ever known to mankind. It is found in three main phases; Aragonite, Calcite and Veterite. Though all the three forms are chemically same but they differ in other aspects like homogeneity, whiteness, purity and thickness. $CaCO_3$ can be extracted by mining or chemically synthesized in the laboratory and is widely used for many industrial purposes, as a biomaterial for tissue engineering applications; drug delivery system, bone tissue engineering and bone grafting [5].

Experimental Methodology

For the preparation of cockle shell $CaCO_3$ powder, approximately 600 grams of cockle shells were first washed by normal tap water to

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remove dirt from the outer surface and inside of the shells. The shells were then boiled for about 30 minutes using a steel container and stove followed by drying in oven (Memmert, Germany) at 60°C.

Diluted sulphuric acid (H_2SO_4) was prepared with a ratio of 15:85 (15% sulphuric acid and 85% distilled water) in a 1000ml beaker. The dried cockle shells were washed in the presence of acid with a cleaning brush to remove the remaining stains from the surface. The shells were dried again in oven (Memmert, Germany) at 60°C for 3 days.

Upon drying, the shells (in white color) were crushed into powder of small grains using a cutting mill (Retch, Type SM 100, 1500W, 230V and 50Hz). The powder was crushed again using a rotor mill (Retch, Type ZM 200, 1100W, 230V and 50/60 Hz) to obtain a fine powder of $CaCO_3$, which was further characterized using scanning electron microscopy (SEM), energy dispersive X-ray (EDX), X-ray diffraction (XRD and FT-IR analysis. The obtained powder was stored in small airtight bottles to avoid absorption of any moisture.

SEM and EDX Analyses: Few milligrams of cockle shell $CaCO_3$ powders were affixed to a stub with carbon paint placed on the sample holder. The sample holder was then fixed on a rotatable disc inside the machine and the cockle shell $CaCO_3$ powders were ready for both SEM and EDX analyses. The surface morphology of the powder sample was observed on SEM (FEI Quanta 400F) operated under low vacuum at an accelerating voltage of 15kV and a current of 60-90 mA. The same sample was also studied for EDX to investigate the elemental constituents as well as the atomic and weight percentage of the elements.

XRD analysis: Firstly, the sample holder was thoroughly cleaned and the cockle shell $CaCO_3$ powder was spread on the sample holder. The sample holder was then placed inside the XRD machine (X'Pert PRO) and the sample was investigated to study the phase(s) of the $CaCO_3$ powder.

FT-IR analysis: The cockle shell $CaCO_3$ powder was mixed with KBr at the ratio of 1:200. The mortar and pestle was thoroughly cleaned with acetone and the mixture of $CaCO_3$ powder and KBr was crushed. The $CaCO_3$ powder and KBr mixture was then put into a die and pressed at a pressure of 6000 Pa using a pressing machine to prepare a transparent disc. The disc was placed on a holder which was later placed inside the FT-IR spectrometer (Spectrum RX I) machine to investigate the unknown materials present in the sample.

Results and Discussions

Scanning electron microscopy

The crystal size and surface morphology of the cockle shell $CaCO_3$ powder prepared was examined by 'FEI Quanta 400F' scanning electron microscope operating at 20 kV accelerating voltage. The cockle shell $CaCO_3$ powder is a non-conducting powder therefore the SEM images were taken at low vacuum mode to obtain sharp images. The images are shown in Figure 1. Figure 1a proved that the cockle shell $CaCO_3$ powder was successfully grinded into fine powder grains. Figure 1b and 1c revealed that the prepared cockle shell $CaCO_3$ powder has rod-like or its cluster structures. These rods/needles like structures were typical aragonite.

Figure 1b was taken at 5000x magnification and showed rod-like particles with a length of about 20 µm whereas Figure 1c was taken at 2500x magnification. The SEM results obtained from cockle shell $CaCO_3$were compared with that of the commercial $CaCO_3$. The commercial $CaCO_3$ powder was examined by LEO 1455 variable pressure scanning electron microscope after coating the sample with gold. The image is shown in Figure 1d which depicts the morphology and crystal structure of commercial $CaCO_3$. Micron sized cube-like calcite crystals were seen in commercial $CaCO_3$ [6].

Calcite and aragonite possess different crystal growth patterns and crystal structure. Aragonite is orthorhombic and calcite is hexagonal. From the crystal structure, it is considered that the nuclei formation of calcite is more difficult as compared to aragonite.

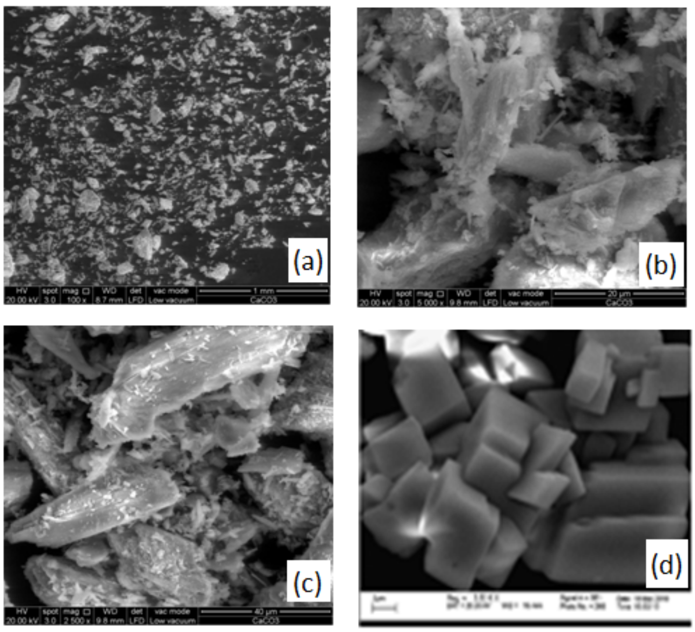

Figure 1: SEM images of CaCO3 powders; (a) Cockle shell $CaCO_3$ powder (Mag. 100x). (b) Cockle shell $CaCO_3$ powder (Mag. 5000x), (c) Cockle shell $CaCO_3$ powder (Mag. 2500x) and (d) Commercial $CaCO_3$ powder (Mag. 3500x).

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Rod-like orthorhombic crystals are unstable as compared to cube-like crystals which makes them preferable for bone regeneration applications.

Energy dispersive X-ray analysis

EDX investigation was carried out at three different spectrums of the sample. The elemental constituents of the synthesized cockle shell $CaCO_3$ powder was investigated at each spectrum. Spectrum 1as shown in Figure 2a revealed that most of the expected peaks looked like C K, O K and Ca K. The percentage of carbon and oxygen was also higher as compared to calcium. In additional to these peaks, distinct peak of Al K was also seen in the first spectrum having an atomic percentage of 0.17. Spectrum 2 was recorded from a different area which showed peaks of C K, O K, Ca K and Al K with another additional peak of Na K as shown in Figure 2b having an atomic percentage of 0.28. Spectrum 3 is shown in Figure 2c where a new distinct peak of S K was observed, having an atomic percentage of 0.25.

As sea water contains sodium (Na), the element sodium is also found in cockle shell $CaCO_3$ powder. The results obtained from EDX were compared with the previous research works on cockle shells (*Anadara granosa*) and it was concluded that Malaysian cockle shells normally have Carbon (C), Oxygen (O), Calcium (C), Magnesium (Mg), Potassium (K), Iron (Fe), Silica (Si), Zinc (Zn), Copper (Cu), Nickel (Ni), Sodium (Na), Boron (B) and Phosphorus (P). Therefore, it is reasonable to have sodium and metallic elements in the synthesized cockle shell $CaCO_3$ powder [7] (Table 1).

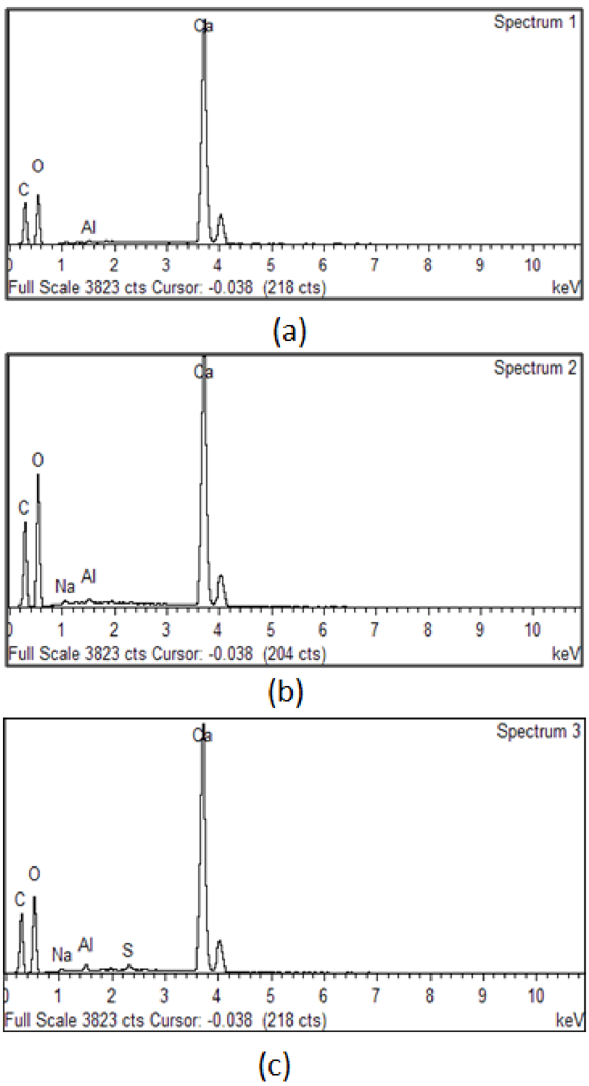

Figure 2: EDX spectra showing the peaks of various elements present in the $CaCO_3$ sample; (a) Spectrum 1, (b) Spectrum 2 and (c) Spectrum 3.

(a)

Spectrum	C %	O %	Na %	Al %	S %	Ca %	Total
1	26.84	54.36	-	0.17	-	18.63	100
2	28.46	59.41	0.28	0.19	-	11.66	100
3	28.9	55.07	0.28	0.33	0.25	15.17	100
Mean	28.1	56.3	0.28	0.23	0.25	15.15	100
Standard Deviation	1.08	2.73	0	0.08	0	3.48	
Maximum	28.9	59.41	0.28	0.33	0.25	18.63	
Minimum	26.84	54.36	0.28	0.17	0.25	11.66	

(b)

Spectrum	C %	O %	Ca %	Cu %	Sn %	Total
1	12.72	48.77	37.47	0.22	0.82	100
2	12.75	53.25	32.99	0.59	0.42	100
3	10.25	42.93	45.78	0.52	0.52	100
Mean	11.91	48.32	38.75	0.44	0.59	100
Standard Deviation	1.43	5.18	6.49	0.2	0.21	
Maximum	12.75	53.25	45.78	0.59	0.82	
Minimum	10.25	42.93	32.99	0.22	42	

Table 1: Percentages of the elemental contents in $CaCO_3$ powders. (a) experimental cockle shell $CaCO_3$ powder, (b) commercial $CaCO_3$ powder.

Table 1a shows the atomic percentages of the elemental contents of cockle shell $CaCO_3$ powder. The table was tabulated from the results determined by EDX. Table 1b shows the atomic percentages of the elemental contents of commercial $CaCO_3$ powder, which is extracted from the previous research work by Islam et al. [6]. It can be deduced that the cockle shell $CaCO_3$ powder has high percentage of carbon and oxygen as compared to the commercial $CaCO_3$ powder. For spectrum 1, the atomic percentage of carbon and oxygen calculated in the cockle shell $CaCO_3$ powder was 26.84% and 54.36% respectively, whereas, in commercial $CaCO_3$ this percentage was calculated only to be 12.72% and 48.77%. Similarly, in spectrum 2 and spectrum3 the atomic percentages of carbon and oxygen in cockle shell $CaCO_3$ powder was calculated to be more than that of commercial calcium carbonate.

X-ray diffractometry

The structure of the cockle shell $CaCO_3$ powder was demonstrated by 'PANalytical X'Pert Powder' XRD machine (Position 2θ, range 20-60). The XRD pattern is shown in Figure 3a. Although there were some unidentified peaks (U.P) observed in the pattern but in contrast with the JCPDS files library 00-041-1475 (Aragonite), most of the peaks obtained in the XRD pattern clearly illustrated the peaks corresponding to h k l of 111, 021, 121, 012, 200, 031, 112, 130, 211, 220, 221, 041, 132, 113, 231, which proved that the phase of cockle shell $CaCO_3$ powder was aragonite. The strong and sharp peaks showed that the cockle shell $CaCO_3$ powder was well crystalline. The crystal size was also measured by using Scherrer's equation [8,9] and the raw data obtained.

$$\text{Thickness} = \frac{K\ddot{e}}{\beta cos\grave{e}}$$

K = shape factor, λ = x-ray wavelength, β = FWHM of diffraction peak, θ = Bragg angle.

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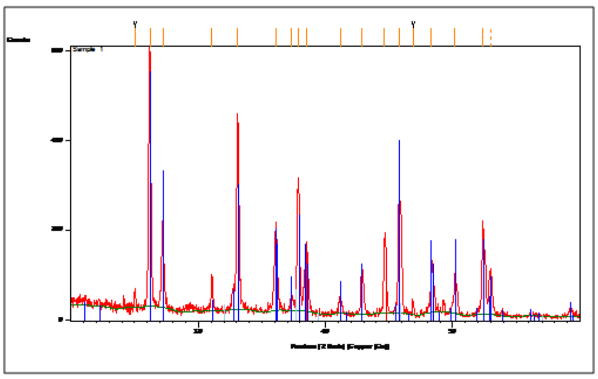
The Scherrer's equation was applied to the peaks (111), (200) and (221) and the grain size was calculated to be 39.64 nm. The obtained XRD pattern was also compared with the standard aragonite XRD pattern shown in Figure 3b [10]. It was concluded that all of the peaks obtained in the XRD pattern of cockle shell $CaCO_3$ powder matched perfectly with the standard aragonite pattern.

XRD offers an easy way to distinguish between the two $CaCO_3$ polymorphs; aragonite and calcite. Both the minerals have their highest intensity peaks at different positions. The highest peak of aragonite is at (111) as seen in Figure 1a whereas the highest peak of calcite is at (104). The aragonite polymorph of $CaCO_3$ is meta stable under all type of geological conditions and is known to have high mechanical strength [10]. That is why aragonite is considered to be suitable for different tissue engineering applications compared to calcite because calcite is more stable compared to aragonite. Aragonite may convert to calcite at 380-470°C temperature.

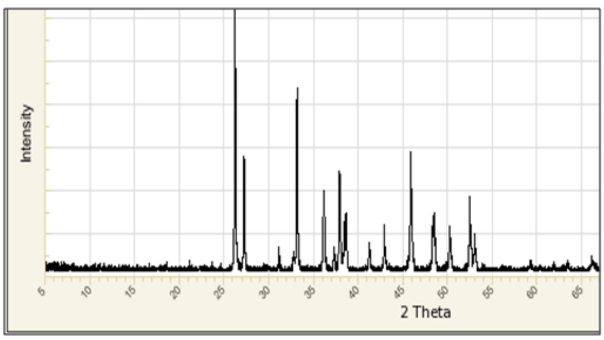
Fourier transform infrared analysis

The phase of the cockle shell $CaCO_3$ powder was further confirmed by 'Perkin Elmer Spectru' FT-IR machine. The obtained spectrum is shown in Figure 4. The spectrum showed vibrational bonds at 1083 cm^{-1} and 861.93 cm^{-1} that can be attributed to the characteristic aragonite symmetric stretching vibration of carbonate and external plane bending vibration of carbonate respectively.

The external plane bending vibration mode is infrared active for all of the three polymorphs; aragonite, calcite and veterite. For aragonite, this vibration mode appears at 861.93 cm^{-1}, as seen in the spectrum whereas for calcite and veterite this vibration mode was observed nearby 876 cm^{-1} and 870 cm^{-1} respectively. Table 2 lists the different wave numbers of functional group in the three polymorphs [11].



(a)



(b)

Figure 3: XRD patterns of (a) synthesized cockle shell $CaCO_3$ powder and (b) standard aragonite.

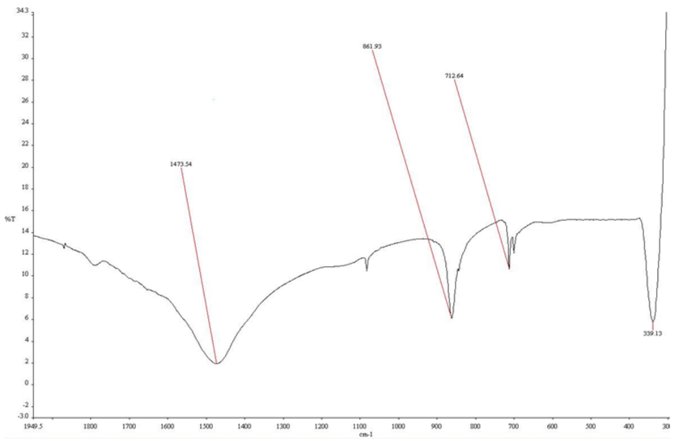


Figure 4: FT-IR spectrum of cockle shell $CaCO_3$ powder.

Phases	Anti-symmetric stretching vibration of C-O	Symmetric stretching vibration of CO_3^{2-}	External plane bending vibration of CO_3^{2-}	Internal plane bending vibration of O-C-O
Aragonite	1421	1082	853	705,700
Calcite	1421	1082	876	713
Veterite	1421	1082	870	750

Table 2: Wave numbers of functional groups.

A sharp peak at 861.93 cm^{-1} confirmed that the cockle shell $CaCO_3$ powder is pure aragonite. The peak at 712.64 cm^{-1} can also be attributed to the internal plane bending mode of aragonite [12]. Another interesting feature was also observed in Figure 4, a pair of bonds at 712.64 cm^{-1} and 700.10 cm^{-1}, which were modes of rod-like aragonite.

It can be seen from Figure 4 and Table 2 that the functional group in the spectrum is aragonite. It can also be proved that no traces of calcite or veterite exist in the cockle shell $CaCO_3$ powder.

Conclusions

The $CaCO_3$ bioceramic powder was successfully synthesized from the cockle shells and characterized using SEM, EDX, XRD and FT-IR. SEM examination demonstrated that the cockle shell $CaCO_3$bioceramic powders had rod-like crystals which is expected to have better resorption and degradation rate as compared to cube-like crystals. XRD analysis showed that the cockle shell $CaCO_3$ powder had aragonite phase and being less stable would have higher degradation as compared to more stable calcite phase. EDX analysis revealed that the cockle shell $CaCO_3$ bioceramic had more carbon and oxygen as compared to commercial $CaCO_3$. FT-IR analysis confirmed that the cockle shell $CaCO_3$ bioceramic powder had the characteristic peak of carbonate group at 1473.54 cm^{-1} and that of aragonite at 861.93 cm^{-1}. FT-IR analysis also evidenced that the powder consisted of $CaCO_3$ itself. In conclusion, the Malaysian cockle shell derived calcium carbonate ($CaCO_3$) bioceramic holds high potential for bone tissue engineering application.

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