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Mineral and Physiochemical Evaluation of Cockle Shell (*Anadara granosa*) and Other Selected Molluscan Shell as Potential Biomaterials

(Penilaian Unsur dan Fisiokimia Kulit Kerang (*Anadara granosa*)
dan Kulit Mollusk Lain sebagai Biobahan yang Berpotensi)

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ABSTRACT

*Molluscan shells are attracting research interest due to the diverse application properties possessed. As shells are very similar to bones, this study was conducted to analyze the mineral and physiochemical composition of Cockle (*Anadara granosa*) shell and three other types of molluscan shell, namely* Strombus canarium, Oliva sayana *and* Terebra dislocata *as potential biomaterial for bone tissue engineering applications. Approximately 200 g of shells from each species were processed and powdered for the purpose of this study. Carbon was analyzed using the carbon analyzer while minerals and heavy metals through ICP-MS. The phase purity and crystallographic structures of the powders were identified using X-Ray Diffractometer (XRD) while the chemical functionality was determined using the Fourier transform infrared (FTIR) spectrophotometer. The analysis showed that Cockle shells contained higher content of calcium and carbon including varying amount of other minor elements comparatively. However, all four types of shell powders were found to contain below detectable levels of toxic elements. Physiochemical analysis on phase purity and crystallographic structures showed similar characteristics of carbonate group present in all four shell types. A predominantly aragonite form of calcium carbonate was detected in both XRD diffractogram and FTIR spectra for all samples. Our findings demonstrated that different types of molluscan shells have almost similar mineral and physiochemical characteristics and a predominantly aragonite form of calcium carbonate that provides a strong basis for their use as a potential bone tissues engineering material.*

Keywords: Anadara granosa*; aragonite; bone tissue engineering; calcium carbonate;* Oliva sayana*;* Strombus canarium*;* Terebra dislocata

ABSTRAK

*Sifat cengkerang molusk yang menyerupai ciri struktur tulang telah menarik minat ramai penyelidik. Kajian ini telah dijalankan untuk menganalisis komposisi unsur dan struktur kimia kulit kerang (*Anadara granosa*) dan tiga jenis spesies molusk lain,* Strombus canarium, Oliva sayana *dan* Terebra dislocata *sebagai bahan asas biomineral untuk aplikasi kejuruteraan tisu tulang. Kira-kira 200 g kulit cengkerang daripada setiap spesies telah diproses dan dianalisis untuk kandungan karbon menggunakan penganalisis karbon dan kandungan unsur dan logam berat melalui kaedah ICP-MS. Ketulenan fasa dan struktur kristalografi telah dikenal pasti menggunakan pembelauan sinar-X (XRD) manakala struktur fisiokimia telah dikaji menggunakan spektrometer transformasi Fourier inframerah (FTIR). Analisis karbon dan unsur menunjukkan kulit kerang (*Anadara granosa*) mempunyai kandungan kalsium dan karbon yang paling tinggi serta unsur-unsur lain dalam kuantiti yang kecil berbanding spesies lain. Unsur-unsur toksik yang dikaji didapati berada pada paras yang diklasifikasi sebagai tidak dapat dikesan dalam kesemua empat jenis spesis yang dikaji. Analisis fisiokimia terhadap ketulenan fasa dan struktur kristalografi menggunakan XRD dan FTIR menunjukkan kehadiran kumpulan kalsium karbonat dalam bentuk aragonit pada kesemua jenis kulit cengkerang yang dikaji. Hasil kajian ini menunjukkan bahawa kandungan unsur dan ciri fisiokimia kulit cengkerang mollusk adalah hampir serupa antara spesies dengan struktur kalsium karbonat dalam bentuk aragonit yang memberikannya kelebihan untuk diguna sebagai bahan asas dalam kejuruteraan tisu tulang.*

Kata kunci: Anadara granosa*; aragonite; kejuruteraan tisu tulang; kalsium karbonat;* Oliva sayana*;* Strombus canarium*;* Terebra dislocata

INTRODUCTION

Molluscan shells have been a subject of research interest in various fields of study including food chemistry (Chang et al. 2007), biotechnology, material sciences, biomineralization (Zakaria et al. 2004) as well as biomaterial engineering (Kim & Park 2010). Research interest in molluscan shells exists since the late 1780's focusing mainly in anatomical and structural studies as well as ecological implications. Advancement in science and technology has opened up wider avenues for material studies in which

studies have started focusing on the organics of the shells through histochemical and biochemical analysis. The abundantly available and the various classes of molluscan species make them an interesting candidate for potential biomineralization studies.

Shells have been shown to represent superior mechanical strength compared to other composite materials (Weiner & Addadi 1997). Such properties displayed include fracture toughness, stiffness and tensile strength. The complex architecture and involvement of biological macromolecules thus contributes to the superiority of a molluscan shell. These external calcareous shells that are microlaminated composite of mineral and biopolymers typically present in a considerable portion and size rendering them useful for human applications (Vecchio et al. 2007). Shells also present an exceptional nanoscale precision and strength 3000 times greater than mineral crystals (Sudo et al. 1997). Two major phases of $CaCO_3$ are known to be present in a shell structure which is aragonite and calcite. Identification of these substance in shells have widely attracted research interest to use shell based materials as a potential inorganic precursor for induction of bone mineral such as hydroxyapatite formation (Kim & Park 2010).

The cockle belonging to the species of Anadara granosa is a type of sea molluscan widely consumed in South East Asian countries in various delicacies. The shells represent a large portion of waste products after the mussels were consumed. Studies by Awang-Hazmi et al. (2007), Islam et al. (2012) and Zakaria et al. (2004) have shown the potential use of the cockle shell based calcium carbonate powder as a source of biomineral for bone tissue applications. The powder obtained from the shells nacreous materials are shown to possess high similarities with coral exoskeletons (Awang-Hazmi et al. 2007) that are readily marketed in the form of Coralline HA as a proven grafting source for bone tissues.

To date there is still a lack of data comparing cockle shell compositions to other molluscan shells that potentially could highlight the superiority of the material. Therefore, this study was carried out in order to add to the currently existing data's on cockle shell materials by characterizing it against a few other common molluscan shells of different species with distinctive morphological differences to determine its differences in mineral and physiochemical properties as well as its suitability as a source for biomineralization studies in bone tissue engineering application. In this study, we also report findings on heavy metals for cockle shells that were not previously given.

Materials and Methods

Samples

Four different shell materials were identified for this study. The shells were randomly selected based on differences in species with notable differences in physical and morphological characteristics. The four species chosen for this study includes *Strombus canarium, Oliva sayana, Terebra dislocate* and *Anadara granosa* which were designated as samples A, B, C and D, respectively. All shell materials were purchased from local seashell vendors with samples collected from locations around Selangor, Peninsular Malaysia. Characteristics and appearance of these species are briefly mentioned in Table 1 and Figure 1.

Sample Preparation

The shell powder was prepared according to the method described by Zakaria et al. (2004). Briefly, samples were first washed thoroughly with distilled water and oven dried for three days at 50°C. Two hundred and fifty gram of each shell material was then crushed into smaller pieces with electric cutter before being placed in a Blendor (240V) for blending process. The powder obtained from the blending process was then sieved using a stainless steel sieve to obtain a powder less than 420 μm in size, sterilized for an hour in the oven at 105ºC and stored in McCartney bottles prior to analysis.

Evaluation of Mineral Composition

Mineral composition evaluation was carried out to determine the content of some major and minor elements as well as heavy metal elements. Carbon detection was carried out using a carbon analyzer (LECO CR 412 USA) on approximately 1-2 g of each sample. For detection of other major and minor elements (Ca, Mg, Fe, Cu, Ni, Zn, B, Si and Na) and heavy metals (As, Cd, Hg and Pb) the samples were subjected to a dry ashing process prior to analysis. Approximately 1-2 g of samples were weighed in a porcelain dish and placed in a muffle furnace. The temperature was increased gradually to 500°C and maintained for approximately 5 h until a whitish or grayish ash formed, after which they were taken out and left to cool at room temperature. Once the ash was cooled, 2 mL of distilled water was added to moisturize the samples. This was then followed by the adding of 2 mL of concentrated hydrochloric acid and steamed on a hot plate. Subsequently, 10 mL of nitric acid were added and left to dissolve for an hour in a water bath set at 100°C. These dissolved materials were then placed in a 100 mL volumetric beaker. The porcelain dish was rinsed several times to ensure all dissolved materials were totally collected into the volumetric beaker before being topped up with distilled water up till 100 mL mark. The solutions was then shaken and filtered through a no. 2 filter paper. The solution obtained was further diluted to 1/100× prior to analyzing with an ICP-MS (DRC-E Perkin Elmer) in triplicates.

Characterization Studies

The phase purity and crystallographic structures of the shell powders was identified with a powder X-ray diffractometer (XRD) while the chemical functionality was determined by

TABLE 1. Brief description of the selected shells

Sample	Species	Family	Shell description	Color
A	*Strombus canarium*	Strombidae	Heavy shells with smooth rounded outline with maximum length between 65 and 100 mm	Light yellowish brown or golden
B	*Oliva sayana*	Olividae	Cylindrical shaped shells with short spire and smooth shiny exterior with a varying length between 20 and 40 mm	Brown to greyish exterior with reddish-brown zigzag markings
C	*Terebra dislocata*	Terebridae	Slender augers shaped with average length between 20 and 50 mm and a pointed spire	Vary with exterior bands of pale grey, dark grey or brown
D	*Anadara granosa*	Arcidae	Ovate shaped, thick solid exterior with average size about 50-60 mm long and 40-50 mm wide	White to yellowish brown exterior and white inner layer

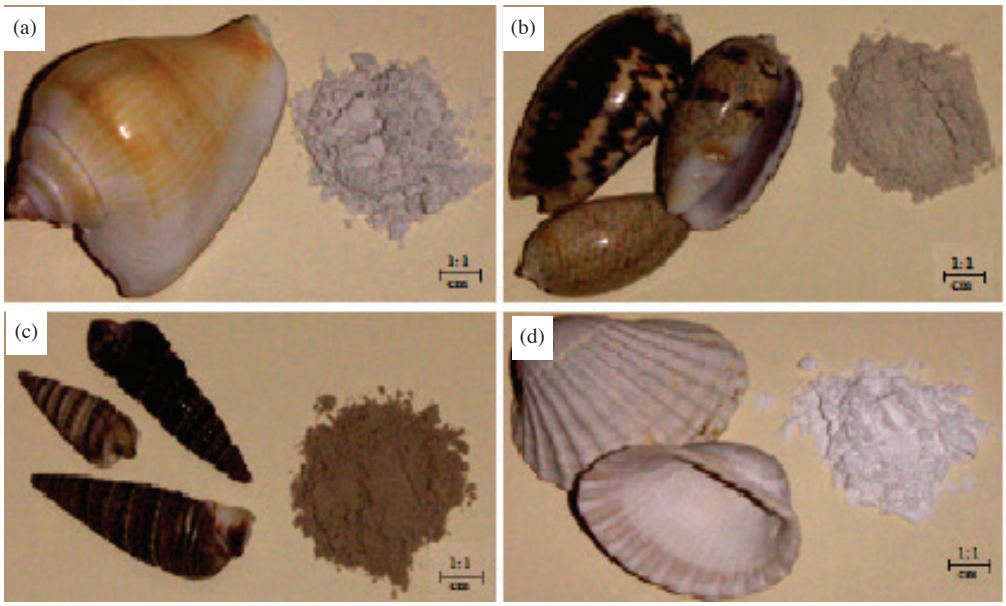

FIGURE 1. Gross and powdered appearances of *Strombus canarium* (a), *Oliva sayana* (b), *Terebra dislocata* (c) and *Anadara granosa* (d)

spectroscopic method using a Fourier transform infrared (FTIR) spectrophotometer (Perkin Elmer) over the range of 400 to 4000 cm^{-1} using 1-2 g of shell powders prepared through UATR methods.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to compare element concentrations in percentages. The results were expressed as mean ± standard deviation (SD). Post hoc test were done for significant values ($p<0.05$) using Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Carbon content was found to be the highest in sample D totaling 11.7% of its composition followed by samples A (11.0%), B (10.3%) and C (9.4%). Table 2 shows the concentration of elements in shells determined by ICP-MS analysis. The element concentration was expressed as part per billion (ppb). However, for the convenience of this study the results were also expressed in percentages (Table 3). Ca and C elements were combined together due to several animal bone studies generally expressing them as one unit (Zakaria et al. 2004) while Cu, Ni, Pb, Mg, B, As, Cd, Si, Zn and Hg were combined together and termed as 'others' based on the fact that the concentrations of these elements were too small. The highest content of CaC was noted in sample D approximately consisting of 96% of total element concentration and was found to be in accordance with the mineral composition of corals as reported in a previous study (Zakaria et al. 2004). This was followed by samples A (93%), B (90%) and C (82%). CaC content of sample C were found to be significantly lower compared with sample D but was found to contain significantly higher amounts of Mg, Fe and other elements compared

TABLE 2. Element concentration in parts per billion (ppb)

Element/ Concentration (ppb)	Sample A (ppb)	Sample B (ppb)	Sample C (ppb)	Sample D (ppb)
Ca	36806.63 ± 476.41	30650.59 ± 4083.43	30785.82 ± 932.93	35453.75 ± 1883.08
Na	1174.63 ± 43.20	878.64 ± 61.88	923.31 ± 26.02	887.10 ± 80.50
Fe	1440.48 ± 54.65	2350.56 ± 32.92	3977.70 ± 76.04	595.46 ± 16.73
Cu	48.40 ± 1.94	BDL	350.43 ± 15.49	93.20 ± 1.62
Ni	BDL	BDL	58.28 ± 0.76	27.07 ± 0.66
Pb	BDL	BDL	24.06 ± 0.45	BDL
Mg	328.75 ± 16.40	680.67 ± 28.53	1999.03 ± 88.06	53.22 ± 2.60
P	2072.15 ± 13.24	370.03 ± 6.54	1629.51 ± 35.82	467.21 ± 2.39
B	BDL	BDL	BDL	BDL
As	BDL	BDL	BDL	BDL
Al	1467.88 ± 16.50	1655.19 ± 24.42	3214.86 ± 21.04	451.26 ± 11.71
Mn	441.13 ± 3.64	47.76 ± 0.60	388.06 ± 8.62	255.38 ± 0.60
Cd	BDL	BDL	BDL	BDL
I	BDL	BDL	58.47 ± 0.70	10.14 ± 0.13
Hg	BDL	BDL	BDL	BDL
Si	91.24 ± 6.99	92.66 ± 2.46	269.01 ± 19.75	± 9.50

*BDL – below detection limits (one to two digits of ppb levels or lower)

TABLE 3. Element concentration in percentages (%)

Samples	Element concentration (%)				
	CaC	Na	Mg	Fe	Others
A	93.0 ± 1.0	2.6 ± 0.1	0.73 ± 0.0	3.2 ± 0.1	0.43 ± 0.0
B	90.3 ± 1.5	2.3 ± 0.3	1.76 ± 0.0[d]	6.1 ± 0.3[d]	0.35 ± 0.0
C	81.7 ± 2.1[a]	2.2 ± 0.1	4.7 ± 0.1[a]	9.3 ± 0.4[a]	2.2 ± 0.2[a]
D	95.7 ± 1.5[b]	2.1 ± 0.2	0.13 ± 0.0[c]	1.4 ± 0.1[c]	0.54 ± 0.0

[a,c]Significantly different from all samples at p<0.05. [b]Significantly different from sample C at p<0.05. [d]Significantly different from samples A and D at p<0.05

with samples A, B and D at $p<0.05$. A significantly higher amount of Mg and Fe were also noted in sample B compared with samples A and D. The lowest amount of Mg and Fe were noted in sample D.

A distinctive feature of the molluscan shells can be attributed to the variation and polymorphism of colours (Sokolova & Berger 2000) that contributes to their morphological differences. From this study it was observed that variation in element concentration in shell materials were contributed by the morphological differences of the species especially its colour. A notable observation of the element contents between the shell materials was that shells that produced a white or off-white powder (samples D and A) upon processing contained higher amount of carbon and calcium as compared with samples B and C which produced a brown and grayish powder, respectively (Figure 1). Lower amount of CaC in samples B and C may be due to the presences of higher content of Fe and Mg which contributed to the varying shades of these shell materials.

It is a well-known fact that various factors pertaining to the environment such as climate and water salinity as well as water pollution level are major contributing factors towards the variations in element contents in shell materials. Despite these factors, only minor differences in element concentration were noted among the different species of shell materials. The mineral content of sample D was also found to be in accordance to the previous study (Zakaria et al. 2004), thus further justifying the potential reproducibility of using shell material for bone tissue engineering.

Heavy metal analyzed as per American society for testing and materials (ASTM), in its F1185-03 2009 report on Standard Specification for Composition of Hydroxylapatite for Surgical Implants requirement includes arsenic (As), cadmium (Cd), mercury (Hg) and plumbum (Pb). All four elements were found to range within one to two digits of

ppb levels or lower thus concluded as below detection limit. These findings are in accord with a study by Chang et al. (2007) on shells of pearl oysters that were found to be safe for human consumption as a food supplement and potential osteogenesis application due to the below detectable levels of heavy metals justifying further the safety of using shell based materials as human implants.

CHARACTERIZATION STUDIES

Regardless of the morphological differences and content of CaC, all the four shell materials showed similar characteristics of $CaCO_3$ form as indicated by the XRD and FTIR analysis. Figure 2 shows the XRD diffractogram of samples A, B, C and D. The XRD analysis shows a predominately aragonite form of $CaCO_3$ in all four samples as compared with a standard calcium carbonate diffractogram with peaks from the shell powders found to be closely matched with the aragonite phase (Joint Committee of Powder Diffraction Society (JCPDS) File No. 00-001-0628).

The FTIR spectra depicted in Figure 3 also showed similar characteristic peaks among the four samples. All the four shell powders showed evidence of presence of carbonate groups. A broad band in the region of 1600 to 1400 cm^{-1} indicated the presence of the carbonate groups of $CaCO_3$ (Lemos et al. 2006). Other functional groups represented by the lesser bands such as amide, amine and phosphates were also noted in most samples but is not discussed in this context.

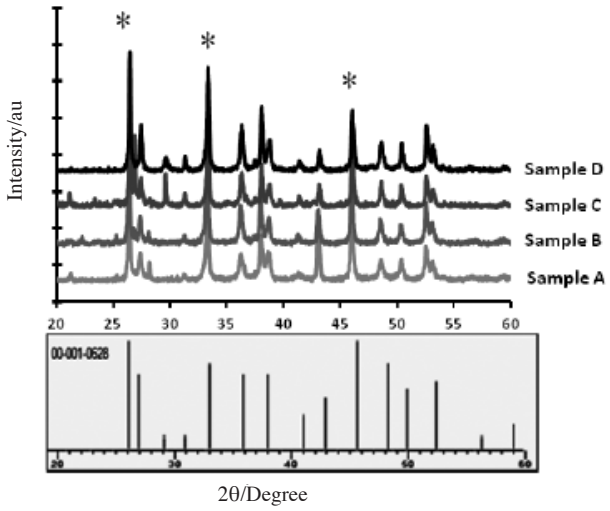

FIGURE 2. XRD diffractogram. (*) indicates dominant peaks corresponding to aragonite form of $CaCO_3$ in all four samples matched with the aragonite phase from Joint Committee of Powder Diffraction Society (JCPDS) file no. 00-001-0628

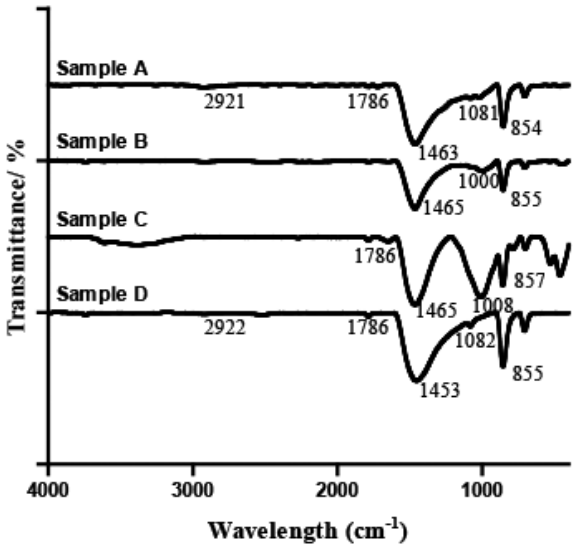

FIGURE 3. FTIR spectra of samples A, B, C and D

First alkene C-H stretching just below 3000 cm^{-1} demonstrates the presence of saturated carbons which can be observed for both samples A and D. These findings also correlate to the higher content of carbon found in these samples as compared with samples B and C. The presence of carbonate groups were indicated by C-O stretching and was found to range between 1600 and 1400 cm^{-1} with 1463.51, 1465.10, 1465.30 and 1453.51 cm^{-1} for samples A, B, C and D, respectively. The results can be compared with a previous study by Fadhlia et al. (2007), which showed C-O stretching of Porites and cortical bone occurring at 1475.58 and 1458.20 cm^{-1}, respectively. This indicates that shell materials despite its differences in species tend to have almost similar characteristics of carbonate groups comparative to bones. The out of plane C-O bending was observed in all the four samples which were between 857 and 854 cm^{-1}. The presence of absorption bands between 1600 and 1400 cm^{-1} and 857 and 854 cm^{-1} in all samples as well as at 1081.92 and 1082.63 cm^{-1} in samples A and D indicate the presence of calcium carbonate in a predominantly aragonite form.

Molluscan shells calcium carbonate compound are known to exist in three different forms namely calcite, aragonite and vaterite, with the latter being least common. Although calcites are a more stable form of calcium carbonate, the aragonite form contributes predominantly to the strength of the shell material. The denser nature of aragonite compared with calcite proves to be an attractive feature for its application in the field of material engineering (Stupp & Braun 1997). An interesting feature of the aragonite mineral is also regarded to its similarities to the human bone density. Aragonite is least produced in laboratories compared to its other crystalline polymorphs and is predominantly occurring in nature as nacreous materials of shells. These calcium carbonate analogs have been shown to be able to integrate and be replaced by natural bones as they resolve (Stupp & Braun 1997). The aragonite form of calcium carbonate from shells is also well regarded as natural ceramic and could bear 3000 times of fracture work compared to pure aragonite (Chen et al. 2008). Studies by Lemos et al. (2006), showed that the transformation of aragonite to hydoxyapatite which features great similarities to the mineralized structure of bones can be accomplished within 24 h, thus further adding to the advantages of using shell based aragonite for bone studies. These features of aragonite could be well manipulated in the field of biomaterial studies as a source of calcium carbonate in order to produce new and improved grafting materials either in the form of bone pastes or bone scaffolds for the ever expanding field of bone tissue engineering.

Conclusion

This study showed that molluscan shells regardless of its species have similar characteristics in regards to their calcium carbonate contents and its phase purity. All the four species of sea shells have similarities in physical properties, but slight differences in chemical contents. The aragonite polymorph of calcium carbonate existing in molluscan shells is an attractive feature that opens up better possibilities in material designs for bone substitutes. The findings in this study showed that the molluscan shells can be used as an organic biomaterial for bone tissue engineering with shell of Cockles showing better potentials due to its higher content of calcium and carbon. The use of nacreous materials obtained from sea shells in the quest for bone replacement materials which are rather cheap and abundantly available worldwide is no doubt a good alternative to corals as a bone replacement material.

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