



Analysis of Scanning Electron Microscopy on *Sinanodonta* (*Anodonta*) *woodiana* Nacre (Lea, 1834)

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Received : 2020-06-01
Accepted : 2020-11-05

Keywords :
SEM, *Sinanodonta* (*Anodonta*)
woodiana, Nacre, Micro structure

Abstract

Chinese pond shell, *Sinanodonta* (*Anodonta*) *woodiana* (Lea, 1834), is a freshwater bivalve that has essential ecological and economic functions. The microstructure of the nacre is of great interest and is the main attraction for the development of pearl farming. This study aims to describe the microstructure and composition of biomineral elements of the nacre at several shell sizes of *S. woodiana*. The shell is cut with a small force on the ventral margin with a size of 3-5 mm for Scanning Electron Microscopy (SEM). SEM images display that a shell layer consists of periostracum, prismatic, and nacre layers. The surface of the nacre layer is irregular or labyrinth patterned. The nacre tablets are hexagonal, glued to each other, so the nacre tablets become polygonal. Moreover, the microstructure of the nacre tablets is like a brick wall and the thickness of tablets from 0.43 μm to 0.59 μm . The composition of the biomineral elements are C, O, Ca, and the mineralization mechanism is under the control of aquatic environmental factors that help the process of microstructure formation in nacre.

INTRODUCTION

Sinanodonta (*Anodonta*) *woodiana* (Lea, 1834) or Chinese pond mussel is a freshwater mussel originating from East Asia, mainly from the Amur river (Heilongjiang) and the Yangtze river (Changjiang). The original distribution areas for this species are the Amur river, Hanka Lake, China, Hong Kong, Taiwan, Kampuchea Thailand, and Japan (Demayo *et al.*, 2012; Chen *et al.*, 2015). This species has been invasively spread to Europe, America, and Indonesia through Cyprinid species (Popa *et al.*, 2011). This type of freshwater mussels of order Unionoida has an important function and contributes to the aquatic ecosystem including biofiltration, food chains, food sources for humans, jewelry, and art

(Vaughn, 2017). Therefore, a study of the use of *S. woodiana* is an alternative in supporting freshwater pearl farming.

Sinanodonta (*Anodonta*) *woodiana* is found in abundance in Indonesia's freshwaters, grows fast, and is capable of producing calcium carbonate (CaCO_3) in the form of aragonite crystals called nacre (Rahayu, 2011). The nacre is produced by bivalves, gastropods, and cephalopods as an internal shell layer. The microstructure of the nacre is of greatest interest as it exhibits a highly organized combination of internal structures, the optical properties that make jewelry so attractive. This is the main reason for the growing development of cross-Pacific pearl farming, especially in Japan, China, the Philippines, Australia,

Cook Islands, Polynesia and Indonesia (Marin *et al.*, 2012).

Many studies have focused on the shell formation of mussels because the nacre layer is structurally similar to the pearl layer. The nacre biomineralization process is of great economic importance to the pearl industry (Liu *et al.*, 2011). The microstructure characteristics of the shells can be used to determine phylogenetic evolution and determine the age of geological formations (Lopes-Lima *et al.*, 2010). Furthermore, Debruyne (2014) explained that the analysis of the microstructure is useful for identifying nacre.

The unique properties of nacre are due to the small proportion (1-5% of weight) of the organic matrix which includes proteins, glycoproteins, chitins, and lipids. This results in a combination of high mechanical strength, similar to ceramics (Southgate and Lucas, 2008; Rousseau and Rollion-Bard, 2012). Nacre is of particular concern for many studies, interesting not only because of its beauty but also because of its strength; despite only 5% of polysaccharide and protein, its work of fracture is up to 3,000 times higher than that of inorganic aragonite (Cartwright *et al.*, 2009). *S. woodiana* can produce freshwater pearls, and sand and gravels in the implantation, producing an increase of pearl layers of 0.57% per day (Lumenta, 2012). At the ideal number of nucleus pearls (2 eggs/mussel with a diameter of 10 mm), the maximum growth is achieved (Rahayu *et al.*, 2013). Furthermore, the presence of a nacre layer

is important information for pearl farming, and consideration of shell selection based on size can be the basis for growing pearls (Lumenta *et al.*, 2017). A lack of research information on the nacre layer on the shells of *S. woodiana* has made the cultivation of freshwater pearls less optimal. Therefore, a study on the description of the microstructure and composition of the biomineral elements of nacre at several shell sizes using SEM is needed. This research can be used as a reference for *S. woodiana* freshwater pearl cultivators so that they can produce good freshwater pearls.

METHODOLOGY

Place and Time

The collection of samples of *S. woodiana* or Chinese pond mussel was carried out on April 2- August 13 2019 in three locations, namely the North Minahasa Freshwater Aquaculture Center (Location 1), Southeast Minahasa Freshwater Fish Seed Center, (Location 2), and the Seed, Pest Control and Fish Disease Center, Minahasa North Sulawesi Province (Location 3) (Figure 1). Sample preparation was carried out at the Laboratory of Fish Health, Environment and Toxicology, Faculty of Fisheries and Marine Sciences, Samratulangi University. Furthermore, the Scanning Electron Microscopy (SEM) test was carried out at the SEM Laboratory of the Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology.

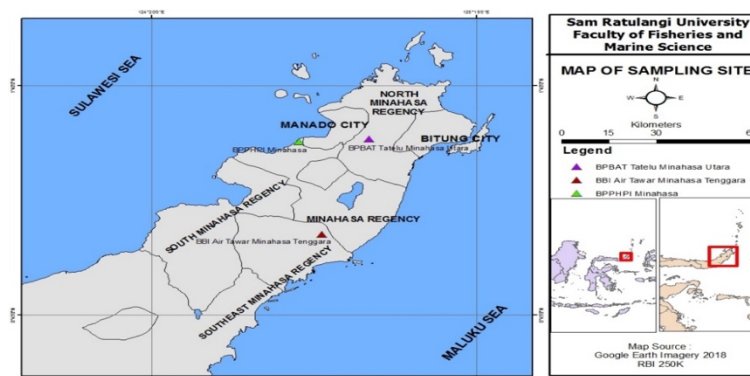


Figure 1. Map of sampling locations.

Research Material

The equipment used in this research included forceps for shell cutting, sputtering, carbon double tape, SEM (JEOL-6510LA) equipped with Energy Dispersive X-Ray Spectroscopy (EDS), containers, and Digital Vernier Caliper (accuracy of 0.01 mm). The material used in this study was a sample of Chinese pond mussel (*S. woodiana*).

Research Design

Scanning Electron Microscope (SEM) is an electron microscope to determine the morphology of *S. woodiana* nacre with magnifications of 200 x, 1000 x and 10,000 x, viewed horizontally and transversally. Shell cut samples measuring 3-5 mm were taken from the ventral margin, placed on the specimen holder using carbon double tape, and then were coated with gold using a sputtering tool. The samples containing the area to be observed were placed in a vacuum to be observed by SEM (JEOL-6510LA) equipped with Energy Dispersive X-Ray Spectroscopy (EDS).

Work Procedures

Samples were transported and put into containers and taken to the laboratory for measurements. The measured morphometric parameters included shell length using the Digital Vernier Caliper

with an accuracy of 0.01 mm. Meanwhile, the measurement of shell length was measured from the anterior end to the posterior end. Samples were sorted based on the size of the shell length (SH) and divided into three categories, namely large (SL > 100mm), medium (SL 80-100mm), and small (SL < 80 mm) sizes.

Data Analysis

The results of observations and measurements in the laboratory were processed descriptively. Images, tabulation, and graphic presentation according to the collected data were the basis for interpretation to describe the characterization of the microstructure and composition of the elements of the nacre.

RESULTS AND DISCUSSION

Sinanodonta woodiana is a freshwater bivalve that lives in rivers, ponds, and another freshwater, characterized by a shell with two valves, symmetrical and connected by a hinge. A total of 36 samples of *S. woodiana* with a shell length of 60-110 mm from different locations were observed by SEM. The SEM results with a magnification of 200 x transversely show that the layer arrangement of the shell consists of three layers, namely the periostracum, prismatic, and nacre layers (Figure 2).

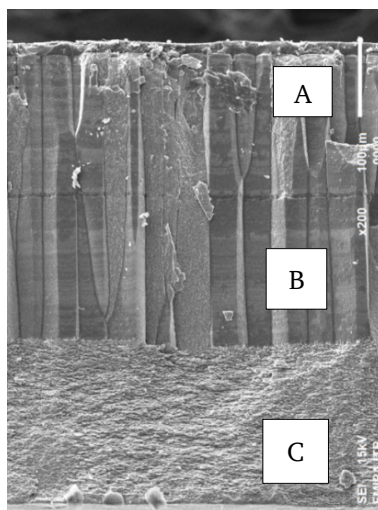


Figure 2. Shell layer structure of *Sinanodonta woodiana* (Lea, 1834).

Note : (A) periostracum layer, (B) prismatic layer, (C) nacre layer (scale bar 100 μ m).

The shell produced by mollusks serves to protect and support the soft body. Generally, it is composed of three layers (from above), namely a thin organic layer called periostracum (Figure 2A). This layer is hard and serves to protect the body from the outside environment such as predators, and serves as a barrier against organisms that contaminate the shell. Furthermore, the periostracum layer is a mineral layer perpendicular to the shell surface. This layer is called the prismatic layer (Figure 2B). This layer is arranged in a long and perpendicular shape between the periostracum and

nacre layers. Then, the third layer (under the prismatic layer) is the nacre layer (Figure 2C). This layer is arranged like a pile of bricks arranged in an orderly manner. The same arrangement is also described by Marin *et al.* (2012) on the freshwater mussel *Unio pictorum*, consisting of periostracum, prismatic, and nacre layers.

Horizontal microscopic observation of the surface layers of the nacre with different shell length sizes (magnification of 200 x) show SEM results with an irregular pattern (Figure 3).

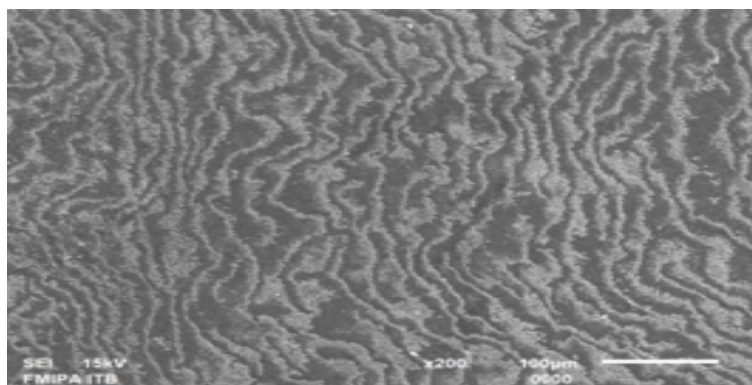


Figure 3. Surface of the nacre (scale bar 100µm).

SEM images show the surface of the nacre layer of *S. woodiana* in an irregular shape like a labyrinth pattern. Cartwright *et al.* (2009) explained that the surface of the nacre layer of several bivalves consists of an arrangement of spirals, targets, and labyrinths.

Furthermore, Liu *et al.* (1999) explained that nacre with a strong iridescent color is caused by diffraction in the nacre layer. The parallel structures and smooth, even surface produce a strong iridescent color in the pearl mussel *Pinctada margaritifera*. The results of this study explain that the irregular surface of

the *S. woodiana* nacre layer is assumed to cause the nacre's color of *S. woodiana* to show less iridescence than that in the pearl mussel *Pinctada sp.*

The surface of the nacre layer observed by SEM (magnification of 1000 x), shows the formation of nacre tablets or aragonite platelets in the direction of growth, and then bonded to one another to form a horizontal layer. The newly formed nacre tablets will glue to other nacre tablets with the help of adhesive. This can be seen in the Figure below (Figure 4).

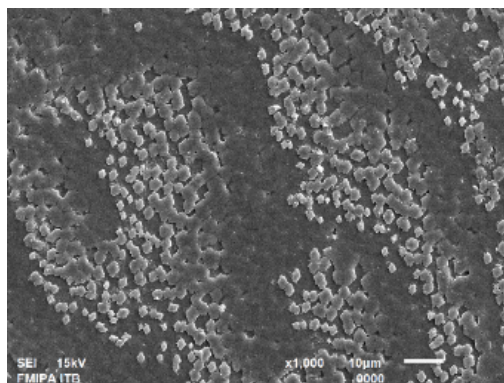


Figure 4. Surface of the nacre (scale bar 10 μ m).

At a magnification of 10,000 x, the SEM image shows the microstructure of a hexagonal nacre tablet covered with a kind of thin adhesive layer to form a unity

with other nacre tablets (like a puzzle). The small, hexagonal-shaped nacre tablets are glued to the larger nacre tablets, so the shape becomes polygonal (Figure 5).

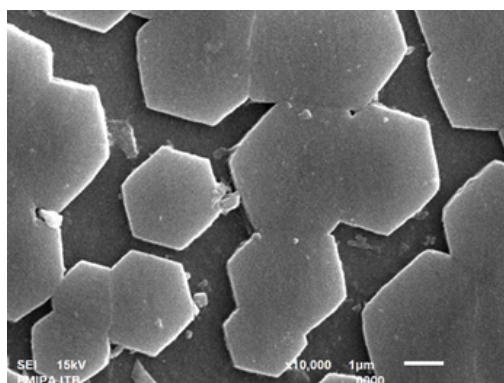


Figure 5. The microstructure of nacre tablets in hexagonal and polygonal shapes (scale bar 1 μ m).

The microstructure of *S. woodiana* is a single, hexagonal crystal, surrounded by an organic matrix. The hexagonal tablets grow with the growth of the nacre to form a uniform horizontal layer. The polygonal shape is the bonding of two or more hexagonal tablets (Figure 5).

Viewed at different locations, it does not show a difference in the shape of the nacre tablets, but the differences in shell length show an irregular shape of the nacre tablets on the smaller shell (<60

mm). Lopes-Lima *et al.* (2010) explained that the observed surface layer of *Anodonta cygnea* nacre is laminal crystals with irregular microstructure in the form of polygonal, hexagonal and rhombic shapes.

From the transverse microscopic observations (1000 x and 10000 x) on nacre, SEM images show that the microstructure of nacre tablets is arranged like a unique brick (Figure 6).

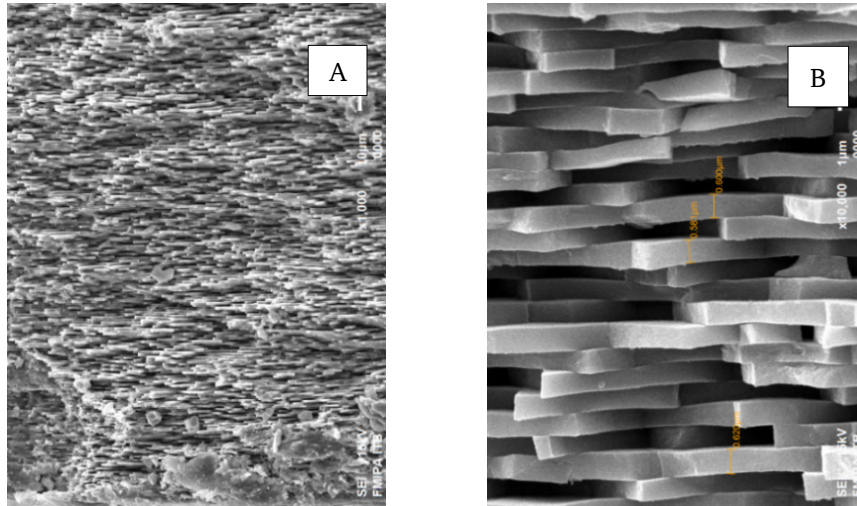


Figure 6. Microstructure of nacre tablets (transversely).
 Where : (A) Scale bar 10µm, (B) Scale bar 1µm.

The types of nacre structure are lamellar/sheet and columnar. The lamellar type is found in bivalves, while columnar can be found in gastropods (Nudelman, 2015). The nacre tablets of *S. woodiana* is a lamellar type, with a neatly arranged brick wall structure. Marin *et al.* (2013) explained that the tablets have a thickness, tightly packed by thin organics to form a layer of uniform thickness, designed to be solid without gaps.

The measurement of the thickness of the nacre tablets at three locations and

three groups of shell sizes from the samples shows variations in the thickness of the nacre tablets. The thickness of the nacre tablets at different locations shows variation between locations. In location 1, the thickness ranges from 0.46 µm - 0.57 µm, in location 2 the thickness ranges from 0.49 µm - 0.59 µm, and in location 3 the thickness ranges from 0.43 µm - 0.53 µm. Overall, the thickness of the nacre tablets ranges from 0.43 µm- 0.59 µm (Figure 7).

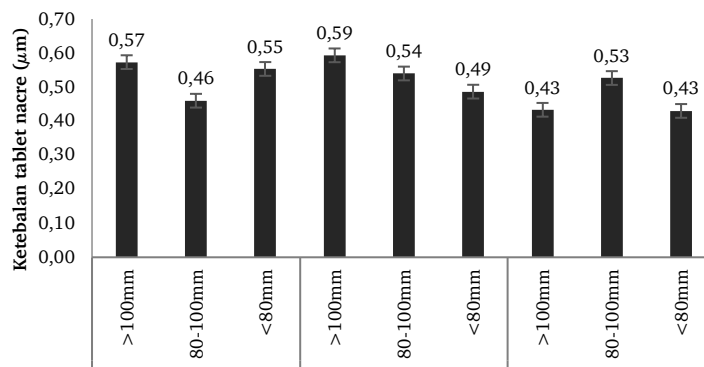


Figure 7. Graph of the thickness of nacre tablets at different locations.

The thickness of the nacre tablets provides information about tablet growth during shell formation in bivalves. Tablet thickness varies on shell size and culture media (Lumenta *et al.*, 2017). The thickness of the nacre tablets at the three locations are different, showing variations between locations and size of shell length (Figure 7). This is due to the

environmental factors of each of the waters that affect shell growth. Water temperature affects the metabolism and feeding activity of bivalves. The filter feeder properties of *S. woodiana* by filtering all organic particles as a food source are affected by temperature. Furthermore, Linard *et al.* (2011) explained that the food chain has an

impact on the ventral and dorsal growth of the shell, and has an impact on the thickness of the nacre tablets in the shells of pearl mussels of *Pinctada margaritifera*. According to Chen *et al.* (2019), the temperature is an important factor affecting the deposition of calcium

carbonate, and pH has a major influence on the solubility of carbonate. In addition, temperature and pH can control calcium carbonate particles that form crystals. Water quality data at the three locations are shown in Table 1.

Table 1. Water quality data.

Parameter	Location 1	Location 2	Location 3
Temperature (°C)	25.2-29.1	25 -28.9	25.2-28.2
pH	7.5-7.9	7.5-8.2	7.3-7.9
DO (mg/L)	4.2-5.9	5.1-5.9	4.5-6.1

This is also supported by Blay *et al.* (2014) stating that nacre thickness correlates with nacre biomineralization. Furthermore, Muhammad *et al.* (2017) explained that the thickness of each nacre tablet is one of the criteria in determining pearl quality because this tablet is a color-causing agent. Pearls with thicker nacre can produce a better shine. This is due to the light infiltrating the nacre, making it a good quality pearl.

The working principle of the Energy Dispersive X-Ray Spectroscopy (EDS) test is to detect X-rays emitted from the samples to characterize the composition of the elements contained. These results are used to quantitatively analyze the mass percentage of the elements contained. EDS analysis of the samples tested provides information on the composition of the main biomineral elements in *S. woodiana* nacre, namely C (Carbon), O (Oxygen), and Ca (Calcium) (Table 2).

Table 2. EDS results on *Sinanodonta woodiana* nacre

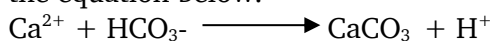
Location	Shell Length	Observation	Mass of Element		
			C (%)	O (%)	Ca (%)
Location 1	>100mm	Horizontal	22.53	47.12	30.15
		Transversal	25.96	47.13	26.91
	80-100mm	Horizontal	23.75	49.31	26.95
		Transversal	32.64	42.83	24.53
	<80mm	Horizontal	24.42	49.46	26.12
		Transversal	32.64	44.16	23.20
Location 2	>100mm	Horizontal	24.17	51.68	24.16
		Transversal	30.37	44.26	25.37
	80-100mm	Horizontal	24.21	50.41	25.39
		Transversal	31.40	45.69	22.92
	<80mm	Horizontal	24.32	49.54	26.14
		Transversal	27.40	46.19	26.41
Location 3	>100mm	Horizontal	22.33	50.35	27.31
		Transversal	23.67	47.06	29.28
	80-100mm	Horizontal	21.43	49.24	29.33
		Transversal	22.35	46.47	31.18
	<80mm	Horizontal	23.03	48.97	28.00
		Transversal	29.50	45.24	25.26

Mollusk shells represent a basic pattern of biologically controlled mineralization, and nacre consists of over 95% calcium carbonate in aragonite crystals, and 1-5% organic matrix

(Lowenstam and Weiner, 1989). Furthermore, Ballesta-Artero *et al.* (2018), explained that the microstructure properties and biomineral elements can reflect the environment in which bivalves

live. Also, environmental parameters can affect the microstructure of the shell.

The biomineral composition of *S. woodiana* is identified to be Ca (calcium) element, which is known as a compound of CaCO₃ (calcium carbonate) and the main constituent of bivalve shell elements. According to Marin *et al.* (2012), calcium carbonate production occurs according to the equation below.



Minerals from the environment can reach the site of calcification through the gills, intestines, or direct absorption by the mantle epithelium. The difference in the chemical properties of the waters where *S. woodiana* lives affect the formation of the shell calcification process. The mineralization mechanism in the shell is a cellular process and is under the control of aquatic environmental factors, and nacre is formed from the crystallization of calcium carbonate (CaCO₃).

CONCLUSION

The shell layer of *Sinanodonta (Anodonta) woodiana* consists of periostracum, prismatic, and nacre layers. The surface of the nacre layer is irregular or labyrinth-patterned, and the nacre tablets are hexagonal in shape and glued to other nacre tablets so that they become polygonal. The microstructure is structured like a unique brick wall, and the thickness of the nacre tablets ranges from 0.43 μm-0.59 μm. Meanwhile, the biomineral compositions found in *S. woodiana* nacre are C (Carbon), O (Oxygen), and Ca (calcium). The mineralization mechanism is influenced by aquatic environmental factors, and nacre is formed from the crystallization of calcium carbonate (CaCO₃).

ACKNOWLEDGMENT

The authors would like to thank the North Minahasa Freshwater Aquaculture Center, Southeast Minahasa Freshwater Fish Seed Center, and the Seed, Pest Control and Fish Disease Center, Minahasa North Sulawesi Province, as

well as all parties who have helped the accomplishment of this research.

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