Microstructure Analysis and Chemical and Mechanical Characterization of the Shells of Three Freshwater Snails

Saida Parveen, Anupam Chakraborty, Dipak Kr. Chanda, Soujita Pramanik, Anandamay Barik, and Gautam Aditya*

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ABSTRACT: The shells of freshwater snails are discarded as waste, which qualify as biological materials with prospective multiple uses. To substantiate this proposition, an attempt was made to elucidate the physical and chemical properties of the shells of three freshwater snails, namely, *Belliomya bengalensis*, *Pila globosa*, and *Brotia costula*. The shells were prepared for electron microscopy and assessment of the calcium carbonate content, apart from the Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), energy-dispersive X-ray spectroscopy (EDS), and nanoindentation studies. The results indicated that the calcium carbonate content (*y*) of the shells ranged between 87 and 96% of the total weight (*x*) and complied with a power regression equation: 

\[ y = 0.801x^{1.016}; \quad R^2 = 0.994; \quad r = +0.998; \quad P < 0.001. \]

Observations through SEM depicted different snail species-specific arrangement patterns of calcium carbonate crystals in the diverse layers of shells. The XRD, FTIR, and EDS observations revealed the dominance of the aragonite form of the calcium carbonate crystal in the microstructures of each snail shell with the occurrence of different shell surface functional groups. The Brunauer–Emmett–Teller analysis elucidated the surface textures of shell dust taken from each snail species; in addition, the nanohardness properties indicate the shells as a tough biocomposite exoskeleton. Species-specific variations in the shell morphology, microstructure, and calcium carbonate content were prominent for the three freshwater snails considered for the study. Nonetheless, the physical and chemical properties substantiate that the shells of *B. bengalensis*, *P. globosa*, and *B. costula* qualify as biological materials for sustainable use in various fields including bioremediation, biocatalyst, biomedical applications, and a source of lime. Since the shells of the freshwater snails are discarded as aquaculture waste, subsequent use as a biological material will support the “waste made useful” paradigm in sustainability, both from ecological and economic perspectives.

INTRODUCTION

In freshwater ecosystem, the snails (Mollusca: Gastropoda) carry out multiple functional roles, many of which are attributable to the shell. Apart from mechanical strength and protection, the shells of freshwater snails facilitate the anchorage of various microbes and periphytons. Among the several features, the shells of freshwater snails are recognized for the complex geometry, architectural peculiarities, diversified color, satisfactory mechanical properties, and the calcium carbonate content. A large number of existing works based on chemical compositions and physical properties substantiate the molluscan shells, inclusive of gastropod shells, as effective composites and toughest biological materials to protect the soft body parts. As the toughest biological materials, the molluscan shells are often preserved in the fossils and specify the stratigraphic age of geological formations. As an extension, evidences taken from structural similarities as well as uniqueness in formation strategies are used to determine the phylogenetic evolution of molluscs. To investigate the relationship between texture and the morphological elements and to correlate the phylogenetic relationship among the connected groups of molluscan species, microlevel structure analysis is obligatory. Biodeposition of minerals on the biological matrix and different orientation patterns to build up the shells of molluscs are directly controlled by physical and genetic factors. Cumulative regulation usually determines the interactions between minerals and organic matrices and other microstructural aggregates. These studies portray the self-assemble microstructures of the molluscan shell and facilitate the understanding of the complex arrangement pattern of the
inorganic and organic chemical components on simple physical ground. An elaboration of the physical and mechanical properties of the gastropod and bivalve shells is therefore essential to corroborate its prospective use in various industrial fields.

The multiple uses of the molluscan shell as a biological material are recognized, which are mostly linked with mussels, clams, abalones, and oysters that are of marine origin.\(^\text{26-29}\) In contrast, the potential of the shells of freshwater snails and mussels as biological materials are yet to be significantly explored. Although they are harvested as food resource,\(^\text{30}\) the efficacy of the discarded shells of freshwater snails and mussels as metal biosorbents appears to be considerably higher\(^\text{31-36}\) in terms of metal removal and availability in nature. The shells of the freshwater snail *Pomacea canaliculata* is used as PVC composite materials\(^\text{37}\) as well as for metal bioremediation.\(^\text{38}\) As a biocatalyst, the processed shells of freshwater snails are used for biodiesel production.\(^\text{39-41}\) The hydroxyapatite prepared from shells of *Bellamya javanica* using chitosan also provides a prospective use of the shells of freshwater snails.\(^\text{42}\) The prospective orthopedic application of the hydroxyapatite crystal obtained from the shells of freshwater snails is also reported.\(^\text{43}\) Such a varied type of application of the shells of freshwater snails calls for the elucidation of the structural features of the shells for promoting as biological materials. In major instances, the shells of freshwater snails are generated as a waste product following extraction of the meat, which serves as a cheap source of protein for the common people\(^\text{30,44}\) and has value for ethnomedicine as well as conventional medicine.\(^\text{35,46}\) The discarded shells of freshwater snails are ideal for exploration, complying with the "waste made useful" paradigm to make sustainable utility of the natural resources. Therefore, characterization of the waste shells of freshwater snails is a pre-requisite to promote their multiple uses as biological materials. In view of these propositions, an attempt was made to characterize the shells of selected freshwater snail species, namely, *Pila globosa* (Swainson, 1828) (Gastropoda: Ampullariidae), *Brotia costula* (Rafinesque, 1833) (Gastropoda: Pachychalidae), and *Bellamya bengalensis* (Lamarck, 1882) (Gastropoda: Viviparidae). Apart from the role in food security and livelihood, the shell of the snail *P. globosa* is proved to be a heterogeneous catalyst in biodiesel production from used frying oil (soybean oil)\(^\text{47}\) and unused oil also (using the shell of *Pila sp.*).\(^\text{48}\) The hydroxyapatite synthesized using the shells of the freshwater snail *Pila ampullacea* as raw materials is used for the adsorption of bichromate ions.\(^\text{49}\) The shell of the snail *B. bengalensis* is a cost-effective biosorbent for cadmium and bears potential in bioremediation.\(^\text{36}\) While the shell formation is intricately guided by physical and genetic factors, the redundant chemical constituents are the calcium carbonate crystals that vary in different molluscan species.\(^\text{12-14,20-22}\)

- **RESULTS**

Microstructure Observation and Characterization with FE-SEM and EDS. The basic architecture of the shell of a freshwater snail (atypical of Gastropoda) is shown in Figure 1, exhibiting distinct layers. The portrayal of each shell layer at the microlevel was made through observation of the FE-SEM images of both the cross-sectional area and surface to inner layers. The shells were prepared by breaking the shells.
and applying mild acid as etching substance for changeable time to expose the consecutive layers. The microstructures viewed from each surface layer of the shells were described following the conventional terminologies.12,50

The FE-SEM images (Figure 2A–C) of cross-sectional views of the shells of B. bengalensis, P. globosa, and B. costula revealed the structural details of the distinct layers. Various arrangement patterns of calcium carbonate crystals on the biological matrix created different strata of shells, namely, the outermost periostracum layer and prismatic layer inner to that, followed by the middle crossed-lamellar structure and innermost nacreous layer. The description of the shell layers being elaborated in the following paragraphs based on their distinctive morphological features was deduced through the microlevel study on the shell samples of the three gastropod snail species.

**Periostracum Microstructure.** The periostracum is the protein-containing outermost protective layer and partly mineralized organic layer, secreted from the mantle groove involved in molluscan shell formation. Emerging from the mantle edge, the periostracum forms a thin insoluble covering outside the shell surface, which slowly thickens at a later stage consisting of three layers. The outer periostracum layer continues through the entire surface covering the thickened middle layer, which is partially mineralized to form nanospheres or spherules. The nanospheres or spherules are arranged in a microlayer structure and run parallel to the inner periostracum.51 In Figure 3A–C, the external unmineralized proteinaceous layer of each shell of the three freshwater snail species is presented. In each shell sample, mild acid etching on the outer periostracum exposed the vacuolated middle layer of periostracum (Figure 3D–F). This principal middle layer was equipped with rod-like structures. The maturation and stratification of this layer were carried out by vesicular mantle cells. Subsequently, these were transformed regularly from one form to another accompanied by subsequent maturation of periostracum. During this period, vacuoles were formed, filling with mineral granules in the middle layer of the periostracum.52 The innermost layer of thin periostracum was uninterrupted with the prismatic matrix, which widened downward to cover the prisms.17,52 In recent years, the fundamental hierarchical order of calcium carbonate arrangement from crystallite columns to lamellae was demonstrated from an archaic mollusc of evolutionary and paleontological importance. In snail shell formation, crystallite column fibers via the formation of lath, folia, and crystalline lamellae assembled into five types of microstructures like crossed foliated lamellar, lamello-fibrillar, foliated aragonite, fibrous foliated, and isolated tablet structures.12,14,17–22,53 In
the present instance, the mineralized zones (viz. prismatic, crossed-lamellar, and nacreous layers) of snail shells showed these types of arrangements following different construction mechanisms.

**Prismatic Microstructure.** Immediately below the organic periostracum layer was the outermost mineralized layer where the CaCO₃ crystals were arranged into a fibrous or columnar structure, which grows perpendicular to the surface of shell. Numerous crystalline domains were surrounded and separated by a thin membrane existing as a prism-like structure. Through its development, this later thickened and formed a meshwork with complete demarcation of individual crystalline domains.²¹ Four types of prismatic layers are common depending on the distinct arrangement pattern. These are simple prismatic, composite prismatic, spherulitic prismatic, and fibrous prismatic.⁵² In the present study, the shells exhibiting various kinds of fibers of different shapes and sizes were piled up in the prismatic layers in different shell samples. The detailed structure (Figure 4A) of the prismatic layer in *B. bengalensis* represented an elongated, lath-like structure³²,⁵³ arranged vertically. The outer surface of the prismatic layer in *P. globosa* was made up of blocky calcium carbonate fibers stacked irregularly (Figure 4B), whereas fibrillar composite prism (Figure 4C) was distinct in the prismatic layer of *B. costula*.

**Crossed-Lamellar Microstructure.** The crystalline laths and, more commonly, the fine prismatic fibers fabricated in aragonite crystal form remain arranged in opposite directions to variable angles and form the interdigitate crossed-lamellar microstructure. The crossed-lamellar structure occupies the middle portion of the shell layers, having higher ceramic content and excellent mechanical performance. Owing to the features of the crossed-lamellar layers, the shells tend to increase in hardness and acquire resistance to fracture.¹³ The crossed-lamellar structure of *B. bengalensis* (Figure 4D) represented the inner radial arrangement of lamellar fibers.¹⁶ The parallel arrangement of first-order lamellae to the plane (of cross-sectional view) was distinct in the crossed-lamellar layer of *P. globosa* (Figure 4E), where the plate-like aragonite crystals pile up to materialize as irregular complex lamellar columns with an orthogonal arrangement. In *B. costula*, the third-order lamellae arranged into a second-order lamellar structure, which compiled further to form a hierarchically irregular complex crossed-lamellar layer (Figure 4F).⁵⁴,⁵⁵

**Nacreous Microstructure.** In general, the nacreous layer, the innermost layer of the shell of the freshwater snails, typically exhibits two kinds of arrangement patterns. The nacreous layer follows either (a) the sheet nacre model, where the individual tablet-like crystalline organization mimics the “brick-and-mortar wall”, or (b) the column stacking model, where crystalline plates stack together vertically to form a pyramidal and columnar arrangement. The crystalline constructions maintain a regular interval throughout the matrix bed to make the biomaterial highly tensile with more accurate bending as well as compressive strength.¹³,¹⁷,⁵⁶

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**Figure 4.** Insight into different mineralization patterns of distinct shell layers of different snail species through FE-SEM images. (A) The distinct arrangement pattern of prismatic layer shows elongated, lath-like thin fibrous prisms vertically arranged in *B. bengalensis*, (B) a vertical alignment of blocky aragonite crystal prisms is available in the outer prismatic shell layer of *P. globosa*, and (C) *B. costula* shows composite fibrillar prisms compiled into an irregular simple prismatic structure. Crossed-lamellar structure of shells of (D) *B. bengalensis*, (E) *P. globosa*, and (F) *B. costula* where nonvertically arranged elongated subunits are stacked in different directions. SEM images of the nacreous layer of (G) *B. bengalensis*, (H) *P. globosa*, and (I) *B. costula*.
As illustrated in Figure 4G, the nacreous layer of B. bengalensis exhibited columns made up of parallel crystalline tablets arranged vertically and stacked perpendicular to each other, forming rows of stacked nacreous secretions. The calcium carbonate unit cell binding through mineral bridges formed tile-shaped plates, which piled up to form a stack-of-coins structure (Figure 5A). This stack-of-coins structure extends laterally to combine further and achieve a mesolayer conformation (Figure 4G). On the growth surface on the successive layers in nacreous region, the interlamellar matrix sheets are secreted first and then the aragonite plates begin to grow between interlamellar matrices, unless they are not confluent properly. In this way, the nacre thickens as the sheets become mature and are added with time. The newly emerged nacreous layer in the growth region on which the aragonite tablets appeared as stacks of coins lies under the layer where the tablets were already nucleated and confluent properly. In this way, the tablets grow in the horizontal direction and the underlying layers are confluent until the layer is closed. The following figures showed the aggregation mechanism of calcium carbonate crystals, which compiled as a stack-of-coins structure scattered on a newly formed nacreous sheet made up of matrix proteins.

In Figure 4H, the vertical pile up of crystalline tablets in the inner nacreous layer of P. globosa represented complex ladder-like growth, compiled in a row stacking manner. Figure 4I depicts the nacreous layer of B. costula where crystal fibers are piled up to follow a typical brick-and-mortar wall model. In addition to the above-mentioned conventional morphological structures of snail shells, a variety of exclusive crystalline arrangements of indecisive mineralogical assembly-created homogeneous structures are spread throughout the different shell layers in a diverse manner.

Stack-of-Coins Structure of Calcium Carbonate Crystal. The stack of coins-like iridescence (Figure 5A) was prominent at the growing regions on the shell nacreous layer of B. bengalensis. This kind of structure is made up of elliptical and pseudo-hexagonal tablets and configured as plates of calcium carbonate crystal. A number of plates lying one above another, forming a coin-stacking structure, which is further distended and has a mushroom-like shape, appear to be enclosed fairly by an organic membrane as circular units. 

Spherulitic Arrangement of CaCO3 Crystal. Continuous growth and regeneration through mineralization are typical characteristics of molluscan shells. The inner surface of the shell is directly involved in the mineralization and synthesis process. Here, the aragonite form of calcium carbonate crystals piles up in needle-like foliates. Such foliates aggregated and diverged from a central point to appear as hedgehog spikes (Figure 5B). These were present on the growth surface and were directly correlated with regeneration events. Thus, the lotus-shaped spherulite aggregates were noticed in the periostracum layer of P. globosa. Although these structures do not form compact shell layers, spindle-shaped crystals together thicken and get intercalated into a solid stellate aggregate, typically facilitating the shell repair and regeneration processes.

Piling of Foliated Plate. The foliated plates of aragonite crystals were aggregated on the depositional surface of the shell of B. costula (Figure 5C). The foliated aragonite crystal plates were stacked one upon another and made horizontal layers, which expanded over the inner growth surface of the shell. This kind of thickened foliated plate was merged and appeared as a continuous sheet of calcium carbonate floating over one another, which is found frequently in the growth region of snail shells.

Elemental Analysis by XRD and FTIR. The comparative XRD patterns of shells of the three freshwater snails (B. bengalensis, P. globosa, and B. costula) revealed similarities in crystalline peaks (Figure 6), confirming the existence of the aragonite form of calcium carbonate. The X-ray diffraction data were acquired in steps of 0.02° at scattering angels (2θ) ranging from 20° to 90°. The XRD phase analysis of aragonite, portrayed in Figure 6, bears high intensity peaks at 2θ = 26.21°, 27.22°, 33.15°, 36.17°, 37.89°, 38.42°, and 45.86° with monochromatic Cu-Kα radiation (λ = 1.5406 Å). The XRD pattern (Figure 6) reflected the intense peaks at (111) and

![Figure 5](https://example.com/figure5.jpg)

![Figure 6](https://example.com/figure6.jpg)
The narrow distribution of pore size is associated with H1-type narrow slit-like pores characterize the H4-type hysteresis loop. Rise to slit pores in the H3-type hysteresis loops, but the respective. The plate-like morphology of the aggregates gave B. costula and showed a H3-type hysteresis loop, while those of B. bengalensis and P. globosa showed H1- and H2-type hysteresis loops, respectively. The estimated pore volume of the shell dust particles of P. globosa and B. costula were 0.043 and 0.031 cm$^3$ g$^{-1}$, with average pore diameters of 3.902 and 3.103 nm, respectively. The values were comparable to the published data on the shell dust of the snail B. bengalensis$^{36}$ (published as 10.143 m$^3$ g$^{-1}$ for the surface area, 0.079 cm$^3$ g$^{-1}$ for the pore volume, and 3.923 nm for the pore diameter). The specific surface area, pore volume, and pore diameter were determined by the Brunauer–Emmett–Teller (BET) method using a Quantachrome Autosorb automated with nitrogen gas (version 4.0). The result revealed that the shell dust particle of B. bengalensis bears the largest pore volume.

BET Measurements. The $N_2$ adsorption–desorption isotherms of the snail shell dust samples displayed in Figure 8 depicted the type V isotherms for B. bengalensis, type III isotherm for P. globosa, and type IV isotherms for B. costula according to IUPAC classification.$^{66-68}$ The shell of P. globosa showed a H3-type hysteresis loop, while those of B. bengalensis and B. costula showed H1- and H2-type hysteresis loops, respectively. The plate-like morphology of the aggregates gave rise to slit pores in the H3-type hysteresis loops, but the narrow slit-like pores characterize the H4-type hysteresis loop. The narrow distribution of pore size is associated with H1-type hysteresis loop.$^{66-68}$ The shell dust of B. bengalensis had an average mesopore size of 33.184 nm, and that of P. globosa had an average mesopore size of 12.667 nm, while that of B. costula had an average mesopore size of 25.156 nm. The BET surface areas of the B. costula and P. globosa shell dusts were 9.620 and 8.430 m$^2$ g$^{-1}$, respectively. The estimated pore volume of the shell dust particles of P. globosa and B. costula were 0.043 and 0.031 cm$^3$ g$^{-1}$, with average pore diameters of 3.902 and 3.103 nm, respectively. The values were comparable to the published data on the shell dust of the snail B. bengalensis$^{36}$ (published as 10.143 m$^3$ g$^{-1}$ for the surface area, 0.079 cm$^3$ g$^{-1}$ for the pore volume, and 3.923 nm for the pore diameter). The specific surface area, pore volume, and pore diameter were determined by the Brunauer–Emmett–Teller (BET) method using a Quantachrome Autosorb automated with nitrogen gas (version 4.0). The result revealed that the shell dust particle of B. bengalensis bears the largest pore volume.

Nanoindentation and Mechanical Properties of the Snail Shells. The load ($P$)–depth ($h$) plots (Figure 9A–C) of all three snail shells of P. globosa, B. costula, and B. bengalensis at different loads (e.g., 100, 300, 500, 700, and 1000 mN).$^{33}$ From the $P$–$h$ plots, it is clearly seen that the final depths of penetrations were the maximum and minimum for the shell samples of B. bengalensis and P. globosa, respectively. So, from the $P$–$h$ plots, it was expected that nanohardness ($H$) would be the minimum for the shell of B. bengalensis and the maximum for the shell of P. globosa. The nanohardness plot in Figure 10A validates that the maximum hardness was exhibited by the shell of P. globosa and the hardness was lower in the case of B. bengalensis. The variation in elastic modulus $E$ of each snail shell sample is shown in Figure 10B. Following these figures (Figure 10A,B), it can be concluded that nanohardness and elastic modulus are load-independent. This is probably
due to the layered structure of the snail shell. In all the snail shell specimens, the ranges of modulus values are $E = 65 - 83$ GPa for shells of *P. globosa*, $E = 55 - 69$ GPa for shells of *B. costula*, and $E = 51 - 53$ GPa in the case of shell samples of *B. bengalensis*. The present results were somehow constant with the nanoindentation for gastropod shells done previously. The arrangement pattern of calcium carbonate crystal plate in a horizontal array is very common and clear in the FE-SEM photomicrograph of each snail shell sample. Due to this layered structure, the nanohardness and elastic modulus showed load independency.

**Characterization and Estimation of Major Element Content of the Snail Shells.**

The representative SEM images and corresponding EDS spectra of the shells of freshwater snails (Figure 11A–C) confirmed the elemental composition, inclusive of CaCO$_3$. The polymorphs of CaCO$_3$ exhibit strong Ca peaks as well as C and O peaks with the incidence of Mg and Si peaks. Upon acid digestion of each shell using 2 M HCl followed by precipitation reaction by adding 2 M Na$_2$CO$_3$, the calcium carbonate content of individual snail shell was estimated. The concentrations of calcium carbonate (mean % by dry weight ± SE) of the shells are as follows: 94.7 ± 1.18 (% *B. bengalensis*), 95.8 ± 4.41 (% *P. globosa*), and 87.2 ± 0.98 (% *B. costula*). Our data somewhat support and validate the previous claim that snail shells are composed of 95–99.9% calcium carbonate. For each species, the CaCO$_3$ content as a function of shell length complied with a power regression equation ($y = 0.05x^{2.860}$, $R^2 = 0.830$ for *B. bengalensis*; $y = 0.104x^{2.885}$, $R^2 = 0.979$ for *P. globosa*; $y = 0.027x^{2.823}$, $R^2 = 0.987$ for *B. costula*) (Figure 12A–C). Irrespective of the three freshwater snail species, the shell weight and CaCO$_3$ content complied with a linear regression equation ($y = 0.801x^{1.016}$, $R^2 = 0.994$) (Figure 12 D). In all instances, strong positive correlations were observed for the shell weight and calcium content for the three freshwater snail species (for *B. bengalensis*, $r = 0.994$, $P < 0.001$; for *P. globosa*, $r = +0.995$, $P < 0.001$; for *B. costula*, $r = +0.998$, $P < 0.001$; for all species, df = 28).

**DISCUSSION**

The gastropod shell is a tough biological material that serves as a protecting exoskeleton of the concerned species. The unique biomineralization process makes the shell stronger, tensile, and resistant to breakage, thereby conferring advantage to the snail to defend against predators and provide anchorage for various biota. In the course of growth and remodeling, each and every layer of the shell is modified in such a way that the shell becomes stronger than the individual sheet. In the present instance, irrespective of the snail species, essential shell-forming building blocks like crossed-lamellar, foliated, and prismatic structures were abundant in each layer of the shells. The basis of these diversified structures can be attributed to the unique crystalline property of
The regular interspacing of crystals in the shells with the organic matrix builds up a rather strong material, with high tensile strength, compressive strength, and bending strength, surpassing qualitatively as biological materials than a number of others. However, such structures and features are redundant in molluscan shells, inclusive of the gastropods and bivalves of marine or freshwater origin.

In a general way, the inorganic part of the molluscan shell is composed of two distinguishable layers: (a) the prismatic layer, covered by the organic periostracum layer and composed of prisms or columns, and (b) the inner nacreous layer composed of nacre lamellae. In addition, a third often distinct (c) crossed-lamellar middle layer is observed for most of the gastropods and mussels. The outermost periostracum, which is itself unmineralized, can effectively take part in the biomineralization process by involving in the prismatic layer formation. The composition and structure of periostracum may favor the enhancement of strength and tensility of the consecutive layers. At a proximate level, the prismatic and nacreous layers of the shell bear structure similarities in both the gastropods and bivalve molluscs, but at deeper and functional levels, species-specific differences are observed. In the case of the freshwater bivalves, particularly, in between the nacreous layer and prismatic layer, there are defined interfacial zones with clear prominence. The outer crystalline layers are the most prominent in bivalves, consisting of various types of either aragonitic or sometimes calcitic prisms. In bivalves, prisms are vertically oriented below the periostracum in a compact arrangement. Such observations are reported for the freshwater mussels, namely, Corbicula bensoni and Lamellidens marginalis. In C. bensoni, a calcite amorphous cloudy middle layer, which may facilitate biomineralization of the shells, floats above the nacreous layer. A distinct nacreous layer with tablets made up of aragonite piled in a parallel elongated row stacking manner is observed in the shell microstructure of C. bensoni. In the case of the shells of L. marginalis, three mineralized layers are present with the columnar aragonite polygonal prisms arranged in a roughly regular interval, forming the prismatic layer. The nacreous layer appears to be present as an aggregate of overlapping aragonite sheets. The aragonite, harder than the vaterite and calcite forms of calcium carbonate, bears versatility in bonding with other elements, which makes the shells composed of aragonite mechanically more stable than the other forms. According to the strength theory of material mechanics, regular interspacing among the nacreous fibers and their vertical pile up helps the biological material in making it highly tensile in bending strength.
In contrast to the sheet-like arrangement in the bivalves, the nacreous layer of various gastropod shells is covered with nacre columns. Each column is composed of a stack of nacre tablets of varying sizes, with a clear hexagonal shape, which is characteristic of the "columnar stacking" model of gastropod shells. The nacreous layers of *B. bengalensis* and *P. globosa* are composed of similar columns compiled in a row stacking manner. In the nacreous layer of *B. costula*, the crystal fibers are piled up in a brick-and-mortar pattern. In addition to the above-mentioned conventional morphological structures, the gastropod shells are featured by a variety of exclusive crystalline arrangements of indecisive mineralogical assembly. Such crystalline arrangements create a homogeneous structure, which spreads throughout the different shell layers in diverse manners.

As revealed through several studies, in the gastropod shell, the prismatic layer, featured by the numerous crystalline domains, takes the shape of a prism, which is surrounded and separated by thin membranes. In the course of growth and development, the structure thickens and forms a meshwork with complete demarcation of individual crystalline domains. In the present instance, the prismatic layer in *B. bengalensis* had an elongated, lath-like vertical arrangement, whereas the outer surface of prismatic layer in *P. globosa* was composed of blocky calcium carbonate fibers stacked irregularly. A fibrillar composite prism was distinct in the prismatic layer of *B. costula*. In most gastropod species, the shell exhibits a crystalline lath and fine prismatic (aragonite) fibers arranged in opposite directions to form the interdigitate crossed-lamellar microstructure in the middle layer. This crossed-lamellar structure of the shells of snails contributes to the higher ceramic content with excellent mechanical performance (often assigned as "ceramic plywood"), which helps the shells in increasing hardness and making it resistant to fracture. In the present instance, the crossed-lamellar structure of *B. bengalensis* showed the inner radial arrangement of lamellar fibers, while in the crossed-lamellar layer of *P. globosa*, the plate-like aragonite crystals appeared to pile up as irregular complex lamellar columns with an orthogonal arrangement. In *B. costula*, the third-order lamellae were arranged into the second-order lamellar structure, which compiled further to form a hierarchically irregular complex crossed-lamellar layer.

A correspondence between the structural uniqueness and the mechanical properties of the shells of *B. bengalensis*, *P. globosa*, and *B. costula* was hypothesized based on the surface microarchitectures of the shells. Validation of this proposition was made through the nanoindentation and elastic modulus studies for each species (shell sample). As revealed in the P–h plots, the final depth of penetrations was maximal for the shell of *B. bengalensis* followed by those of *B. costula* and *P. globosa*. Alternatively, it was validated by the maximum hardness and elastic modulus values for the shell samples of *P. globosa* and minimum values for *B. bengalensis*.

![Figure 12. Calcium content (in mg) in all three species (A) B. bengalensis, (B) P. globosa, and (C) B. costula as a function of shell length (in mm). (D) Correlation between shell weight and CaCO3 content, irrespective of all three species.](https://dx.doi.org/10.1021/acsomega.0c03064)
The mechanical responses of the shell samples can be deduced through the microstructural peculiarities since intricate hierarchical intertwining between organic components and minerals determines the mechanical strength of composite materials. In the case of a molluscan shell, the unique arrangement pattern of crossed-lamellar structure contributes compound interfaces at different planes of lamellae to help in dissipation of energy during crack bridging, deflection of crack, and other deformations. The structure of a unit crystal prism in the prismatic layer may also determine the scratch resistance, abrasion, indentation, and potentiality to resist the plastic deformation. In addition, the variations in the prism fibers of the prismatic layer may also influence the indentation properties. Thus, the variations in the mechanical properties of the shells can be attributed to the species-specific differences in the design of the minerals and organic matter contents. Different orientation patterns of crystal laths within a mineralized layer can influence the mechanical behavior of the entire composite. The column stacking constructions observed in the nacreous layer of P. globosa where crystalline plates stack together vertically to form pyramidal and columnar arrangements are known to provide compressive strength to take the maximum loads. It was also reported earlier that organic layers in shells helps in repairing the structures as well as promoting the mineralization process. It was also observed that the lesser the rate of mineralization, the narrower the mineralized layer coupled with the presence of thick organic layers. The thicker organic layer and lower rate of mineralization could be a possible reason for reducing Young’s modulus in shells of B. bengalensis and B. costula. The organic matrix bed associated with rich protein content and less chitin made the shell soft. Although the microlevel organization of the crossed-lamellar structures for all three species were more or less similar, having elongated crystalline laths arranged in a criss-cross manner, changes in lamellar orientation was observed just below the criss-crossed layer in the case of P. globosa (Figure 4E) where the larger lamellae inclined perpendicular to the outer lamellae in prisms. Changes in crystal orientation make the shell more resistant to fracture. The growth and structural peculiarities of the molluscan shells, particularly the bivalve and gastropod shells, have been studied to corroborate the evolutionary processes, physical properties, and functional roles. The elaboration of the microarchitecture, chemical composition, and mechanical properties has been the foundation for promoting the molluscan shell as a biological material with multiple industrial, agricultural, and biomedical applications. While it is considered as a waste originating from aquaculture or agriculture, the hydroxyapatite derived from the shells of the freshwater snails has biomedical applications. In addition, the shells are used as biocatalysts for biodiesel production as well as in metal bioremediation, thereby justifying the highly cost-effective means of recycling of waste. Thus, to qualify as a potential source for various
industries, the calcium carbonate content of the shell of a snail needs to be substantially high. As observed in the present instance, the calcium carbonate content in the species remained between 80 and 95% for the snails B. bengalensis, B. costula, and P. globosa, depending on the shell weight, similar to other species found elsewhere. In the case of the snail Helisoma trivolvis, the calcium carbonate content varied between 95 and 98% depending on the habitat of the snails (different ponds). For the snails Physa sp., B. glabrata, and Pomacea bridgesii, the calcium carbonate content was more than 98%. In the present instance, the calcium carbonate content of the shells was a positive function of the shell weight, reflecting that the shell calcium content increases with size and age of the snails, irrespective of the species concerned. While the calcium carbonate content of the shells of B. bengalensis as well as other snails is primarily attributed to the metal removal potential through biosorption, the efficacy may improve with the modification to a biocomposite. Thus, further exploration of the shell inclusive of the organic components is required to promote its possible use as a biocomposite. Nonetheless, the present study provides an overview of physical and chemical characteristics of the shells of the freshwater snails to highlight their use based on the waste made useful principle of sustainability.

**CONCLUSIONS**

The shell structures of the three freshwater snails B. bengalensis, P. globosa, and B. costula were prominently visible as the outer periostracum, followed by prismatic, crossed-lamellar, and innermost nacreous layers. In all the snail species, the crystal fibers of the crossed-lamellar structure showed the same pattern of arrangement, like plywood, which enables resistance against spread of cracks, if any. Owing to the unique crystalline property of aragonite, providing constructive flexibility, the building blocks of each layer of the shells were diverse in shape. Various types of functional groups were observed, perhaps linked with the interactions between the crystal and organic matrix. A correspondence with the structural and mechanical properties was deduced through the nanohardness and nanoindentation studies, which revealed species-specific variations. The abundance of calcium carbonate provides evidence of the role of the shells in calcium regulation and carbon assimilation and, therefore, the significance in the global carbon cycle. Thus, encompassing remarkable structural complexity and having numerous adaptive features, the shells of the three freshwater snails qualify as mechanically strong and tough biological materials.

In the present perspective, the shells of B. bengalensis, P. globosa, and B. costula reflected structural and mechanical features in support of their multifunctional roles including heavy metal removal, biocatalyst for energy production, and biomedical usages. In the course of harvest and subsequent consumption of the soft tissues, the shells of these snails are discarded as waste, which can be further used as a biological material, complying with the principles of sustainability and reuse of waste matters. Further characterization of the shells of these and other species of freshwater snails should be carried out to explore the various applications of these bioresources as useful materials.

**EXPERIMENTAL SECTION**

**Preparation of Materials.** The freshwater snails were collected from the selected ponds and lakes of Kolkata and Howrah, India, from time to time between 2017 and 2019 as per the requirement of the experiment by employing an insect net of 200 μm mesh size or hand-picking from the substratum. Following collection, the snails were brought to the laboratory for identification and maintenance. The identified snails were segregated and maintained in the laboratory until death, following which the shells were collected and used in the experiment. In the present study, the shells of the three common freshwater snails of India were used, namely, B. bengalensis (Lamarck, 1882) (Gastropoda: Viviparidae), P. globosa (Swainson, 1828) (Gastropoda: Ampullariidae), and B. costula (Rafinesque, 1833) (Gastropoda: Pachychalidae). For the experiments, the shells of the dead snails were cleared of flesh remnants, if any, while the opercula were removed and discarded. The shells used for the analysis were washed properly under running water and then sun-dried for at least 72 h. Prior to preparation of the shells for further studies, the shell weight (in mg) and shell length (in mm) were measured, irrespective of the species, and recorded for analysis. The shell length and shell weight of the snails were as follows: B. bengalensis (n = 30) shell length, range: 11.14–27.16; mean, 19.44 ± 0.91SE; shell weight, range: 49.9–898.9; mean, 291.93 ± 36.54SE; P. globosa (n = 30) shell length, range: 22.056–63.56; mean, 43.76 ± 2.28SE; shell weight, range: 750.9–17126.6; mean 7127.31 ± 950.45SE; B. costula (n = 30) shell length, range: 15.66 ± 64.44; mean, 38.03 ± 1.67SE; shell weight, range, 98.9–3077.7; mean, 1153.13 ± 114.27SE. At least 10 randomly selected shells of each of the snail species were considered for the microscopic, spectroscopic, and mechanical studies, apart from the 30 shells of each species used for the estimation of the calcium carbonate content. Thus, at least 40 shells of each freshwater snail species were considered for the study.

**X-ray Diffraction (XRD) Study.** Freshly prepared dry snails shells were crushed using a mortar and pestle. The crystalline phase compositions of shell dust of the shells of three different freshwater snails (P. globosa, B. costula, and B. bengalensis) were determined by powder XRD analysis using an X’Pert Pro MPD diffractometer (PANalytical, Almelo, The Netherlands). Phase composition data acquisition was performed by stepwise scanning mode. The data were collected in steps of 0.02° at scattering angles (2θ) ranging from 20° to 90° by using monochromatic Cu-Kα radiation (λ = 1.5406 Å) at 40 kV and 20 mA.

**Fourier-Transform Infrared Spectroscopy (FTIR) Study.** To identify the functional groups, the shell dust samples were characterized by FTIR analysis with KBr pellet in a Fourier-transform infrared spectrometer (Jasco FT/IR-6300typeA; serial no. A014461024). The spectra were collected at a resolution of 4 cm⁻¹ with the scanning speed 2 mm/s over a range of 300 to 3500 wave numbers (cm⁻¹). KBr pellets of shell dust were prepared by mixing 1 wt% snail shell dust with 99 wt% KBr powder and ground in an agate mortar and pestle to transform into uniform consistency. The shell dusts of three different snail species were mixed with KBr powder separately and the ground sample was transferred into a Specac Atlas 15T manual hydraulic press to make distinct pellet of each sample for FTIR analysis.
Field-Emission Scanning Electron Microscopy (FE-SEM) and Energy-Dispersive X-ray Spectroscopy (EDS). Washed snail shells were ground into pieces and, to access the consecutive inner layers, dilute chemical etching, i.e., 0.1 N HCl, was applied for a considerable time so that the successive inner shell layers were exposed gradually. Prepared shells were then coated and mounted by using a 3 nm thin platinum film and was made ready for FE-SEM imaging to gain insight into the surface morphology and consecutive inner layers were achieved through acid etching practice using field-emission scanning electron microscopy (model JEOL JSM-7600F) operated at 15 kV to gain adequate resolution and conductivity for the proper magnification. Chemical composition and elemental characterization of snail shells were carried out using the energy-dispersive X-ray spectroscopy (INCA Energy 250 & HKL Advanced EBSD system) attached to FE-SEM.

Brunauer–Emmett–Teller (BET) Method and Determination of Nanomechanical Properties of Shells. The surface area of ground snail dust was measured by nitrogen adsorption–desorption experiment, which was carried out at 77 K in a BET surface area analyzer (ASIQ MP, Quantachrome, USA; version 4.0). The surface area was estimated from this experiment by adopting the well-known Brunauer–Emmett–Teller (BET) method.66,67

The broken pieces of shell samples were first mounted in a stainless steel mold by using epoxy resin and hardener and kept the whole system for 24 h. After that, the resin-mounted shell samples were taken out of mold, and the samples were ground and polished to make the surface flat, parallel, and mirror-finished. Then, the nanomechanical responses were measured. A nanoindentation machine (Fischerscope H100-XYp; Fischer, Switzerland) was utilized for the evaluation of nanomechanical properties like nanohardness (H) and Young’s modulus (E). A triangular Berkovich nanoindenter was used in the present experiments. The tip radius of the nanoindenter was ~150 nm. The corresponding semi-apex angle was ~65.3°. All nanoindentation experiments were conducted at room temperature on the samples, which had been already polished. Prior to each experiment, the area function of the indenter tip was evaluated. It was carried out by the dedicated software available in the machine. The software also corrected the experimentally obtained load (P) versus depth of penetration (h) data for the tip blunting effect. The machine worked as per the DIN 50359-1 standard. Before each experiment, the machine was calibrated with a BK7 glass block (Schott, Germany) provided by the supplier of the machine, having a nanohardness H of 4.14 GPa and Young’s modulus E of 84.6 GPa. This was done to make sure that the data generated remain reproducible. Next, the well-known Oliver–Pharr model was used to measure the nanohardness and Young’s modulus data of the polished shell samples from the experimentally measured load–depth (P–h) data plots.69 A square array of 6 × 6 indents was made for this purpose for each load. Thus, at least 36 individual measurements of nanohardness values were used for each reported average data. In the present experiment, the load was varied from 100 to 1000 mN. The loading and unloading times were kept constant at 30 s for each experiment.

Estimation of CaCO3 Content in Snail Shells. The CaCO3 content of the shells of the three freshwater snails were measured using varying shell size. At least 50 shells for each species were considered, which varied in size and weight. Initial acid digestion of each shell using 2 M HCl followed by precipitation reaction by adding 2 M Na2CO3 enabled estimation of calcium carbonate content of individual snail shell.77

\[
\text{CaCO}_3 + 2\text{HCl} \rightarrow \text{CaCl}_2 + \text{CO}_2 + \text{H}_2\text{O}
\]

\[
\text{CaCl}_2 + \text{Na}_2\text{CO}_3 \rightarrow \text{CaCO}_3 \downarrow + 2\text{NaCl}
\]

The random selection of 25 shells were made from an initial collection of shells of more than 50 individuals of different size classes forming a randomly selected population of the snails collected from the natural habitats. The shell measurements were taken to the nearest 0.1 mm using a Vernier caliper (Insize, Brazil). After removing the flesh and operculum, individual snail shells were ground, 1 M HCl solution was continuously added, and the samples were stirred at room temperature until full digestion. To eliminate large particles of impurities and shell remnants, the slurry was filtered through Whatman grade no. 1 filter paper. Subsequently, 1 M Na2CO3 was added into the solution until the total precipitation reaction was completed to form CaCO3 and sodium chloride. At the end of precipitation reaction, the product substances were cooled down at room temperature. This solution was further filtered and washed thoroughly under running water to eliminate the soluble salt products associated with CaCO3 precipitate. Filtered CaCO3 procured from each snail shell is collected and dried for quantification. A regression equation103 was constructed to portray the relation between shell length and calcium content of the three snail species.

Author Information

Corresponding Author

Gautam Aditya — Department of Zoology, The University of Burdwan, Burdwan 713104, India; Department of Zoology, University of Calcutta, Kolkata 700019, India; Email: gautamaditya2001@gmail.com

Authors

Saida Parveen — Department of Zoology, The University of Burdwan, Burdwan 713104, India
Anupam Chakraborty — Department of Zoology, University of Calcutta, Kolkata 700019, India
Dipak Kr. Chanda — School of Materials Science and Nanotechnology, Jadavpur University, Kolkata 700032, India; Email: dipakchanda014@gmail.com, orcid.org/0000-0003-0147-4972
Soujita Pramanik — Department of Zoology, University of Calcutta, Kolkata 700019, India
Anandamay Barik — Department of Zoology, The University of Burdwan, Burdwan 713104, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c03064

Author Contributions

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