



Magdalena Kowalewska-Groszkowska^{1*}, Dominika Mierzwa-Szymkowiak¹, Joanna Zdunek²

¹ Museum and Institute of Zoology, Polish Academy of Sciences, ul. Wilcza 64, 00-679 Warsaw, Poland

² Warsaw University of Technology, Faculty of Material Science and Engineering, ul. Wołoska 141, 02-507 Warsaw, Poland

*Corresponding author. E-mail: mkowalewska@miiz.waw.pl

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THE CROSSED-LAMELLAR STRUCTURE OF MOLLUSK SHELLS AS BIOCOMPOSITE MATERIAL

Composites produced in nature, such as mollusc shells, are renowned for their unique structures and exceptional properties. The crystallographic characterization of different shells as well as their physical and chemical properties have always attracted the interest of researchers. Much information is available at present, however, most of it concerns sea molluscs. We focused on the microstructures and chemical composition of the shell of land snails of the *Cepaea* genus. New aspects of the microstructure of shells have been shown through the use of a scanning electron microscope (SEM) equipped with an EDS X-ray detector, and X-ray diffractometry (XRD). The study shows that all the tested snail shells are characterized by a typical crossed-lamellar structure and are built of aragonite. Small differences in the chemical composition of the shells as well as differences in the size of the crystallites and different proportions of the amorphous phase were also noticed.

Keywords: shell, crossed-lamellar structure, chemical composition, phase identification

STRUKTURA SKRZYŻOWANYCH PŁYTEK MUSZLI MIĘCZAKÓW JAKO PRZYKŁAD MATERIAŁU BIOKOMPOZYTOWEGO

Naturalne materiały kompozytowe charakteryzują się unikalną strukturą i wyjątkowymi właściwościami mechanicznymi. Obecnie dostępnych jest wiele informacji na ten temat, ale większość z nich dotyczy mięczaków morskich. W artykule skupiono się na mikrostrukturze i składzie chemicznym muszli ślimaków lądowych z rodzaju *Cepaea*. Opisano strukturę skrzyżowanych płytek muszli tych ślimaków jako przykład naturalnego materiału biokompozytowego. Mikrostrukturę zbadano za pomocą skaningowego mikroskopu elektronowego (SEM), detektora promieniowania rentgenowskiego (EDS) i dyfraktometru rentgenowskiego (XRD). Wykazano, że badane muszle ślimaków charakteryzują się typową strukturą skrzyżowanych płytek i są zbudowane z aragonitu. Zauważono niewielkie różnice w składzie chemicznym, w wielkości kryształitów muszli badanych gatunków, a także w udziale fazy amorficznej.

Słowa kluczowe: muszla, struktura skrzyżowanych płytek, skład chemiczny i fazowy

INTRODUCTION

The phylum Mollusca is one of the largest and most diverse groups of animals. There are over 100,000 living mollusc species (approximately half have been described) and 60,000 fossil molluscs. The phylum Mollusca contains eight classes: Caudofoveata, Solenogastres, Polyplacophora, Tergomya, Bivalvia, Scaphopoda, Cephalopoda, and Gastropoda [1]. Gastropods are the most numerous molluscs and they account for approximately 80% of the total. For many years, the shells of different molluscs have been an inspiration for not only biologists, but also chemists, physicists and material engineers. They investigate shell structures, their mechanical properties, and the overall biochemical reactions which result in the creation of these optimized structures.

The molluscan shell is a highly ordered hierarchical structure and a complex organic-inorganic biocomposite material. They are composed of one or both of the crystal forms of calcium carbonate (aragonite or calcite) and an organic matrix [2-4]. Typically, there is an outer organic layer, the periostracum, forming the outermost portion of the shells [5]. The authors of many publications have found several characteristic combinations of mollusc shell structures: (1) homogeneous (e.g. Bivalvia), (2) prismatic (e.g. Bivalvia), (3) crossed-lamellar (e.g. Polyplacophora, Bivalvia, Scaphopoda and Gastropoda), (4) spherulitic (e.g. Bivalvia, Cephalopoda and Gastropoda), (5) and nacreous (e.g. Bivalvia, Cephalopoda and Gastropoda) [5-13].

Most publications refer to the structure of marine molluscs and less of terrestrial molluscs. Thus, we have undertaken detailed studies describing the crystallographic and chemical characteristics of two species of gastropods from the genus *Cepaea*. Snails in the genus *Cepaea* are among the most famous terrestrial European molluscs, however, little is known about their shell structures or their physical, chemical, or mechanical properties. Many previous studies focused on shell colour and banding patterns. As a consequence of shell polymorphism, *Cepaea*, especially the widespread *C. nemoralis* and *C. hortensis*, have become model taxa for ecogenetic research - the effects of the environment on the genetic structure of populations [14-18].

In this paper is the first study of the structures and the chemical compositions of the shells of two species of the genus *Cepaea* using scanning electron microscopy (SEM), energy dispersive spectrometry (EDS) and X-ray diffractometry (XRD).

MATERIAL AND METHODS

Specimens and basic measurements

In this paper, European snails from the genus *Cepaea* (*C. hortensis*, *C. nemoralis*) were used for shell structure analysis. 60 specimens of the PAS Museum and Institute of Zoology collected in the area of the Kraków-Częstochowa uplands were used to study the size of the shells. Free-living individuals, 10 snails of each species, were found in their natural environment in the period of two years in the locality of Kielce and environs of Częstochowa. We collected adults based on the presence of a 'lip' at the mouth of their shell (this lip indicates that the snail has attained adulthood). The snail's soft body was removed, then the shells were rinsed using distilled water. The shell dimensions were measured to the nearest 0.1 mm using callipers and the weight was measured using a digital analytical balance with an accuracy of 0.1 mg. In this study, the influence of the substrate on the formation and structural-chemical properties of snail shells was not considered. However, it is known on the example of other species that the chemical composition of terrestrial snail shells reflects the environmental conditions in which these snails live [19]. To date, no work has been carried out to determine the impact of the substrate on the construction of *C. hortensis* and *C. nemoralis* shells. On the other hand, the influence of environmental conditions (habitat effect) on the polymorphism of *C. nemoralis* shells was quite well discussed in the literature [15, 16].

Scanning electron microscope

The samples were embedded in resin and polished using various grades of diamond paste. Then, the polished sections were subjected to etching in order to reveal the structure (in 5% formic acid for 30 s) and rinsed with distilled water and air-dried. Surface mor-

phology and cross-section observations were conducted by a scanning electron microscope (Hitachi S-3400N) housed in the SEM Laboratory at the Museum and Institute of Zoology of the Polish Academy of Sciences (Warsaw). The scanning electron microscope Hitachi S-3400N is able to work in three various modes: one of them is the natural mode (Low Vacuum), which utilizes the backscattered electron detector (BSE). Moreover, application of the low vacuum mode permits examination of the sample without any special preparation necessary for non-conducting materials, such as shells. The SEM was operated at 25 KeV and at a 10 mm working distance.

Chemical analysis

The chemical examinations were performed on the scanning electron microscope (Hitachi S-3400N) equipped with an EDS X-ray detector for compositional analysis. Quantitative as well as qualitative analyses were carried out on the X-ray SuperDry II detector and analysed by the Noran SIX System program using the Proza corrective method (Phi-Rho-Z). Measurements were made using a lifetime of 30.0 s and an accelerating voltage of 15 kV. The analysed elements were C, O, Si, Ca, Na, Mg, Sr, Al, S, P, Zn, Fe and Mn. A minimum of 32 analyses was performed at various locations on each polished shell section. These point analyses were averaged to obtain an individual mean.

XRD analysis

5 shells of *C. hortensis* and 5 shells of *C. nemoralis* were used for XRD analysis. Two samples of powder were obtained, one for each species. All the shell samples (as a powder) were analysed by conventional powder X-ray diffractometry (XRD). X-ray diffraction studies on the powder samples were carried out using a high resolution Bruker Advance D8 XRD diffractometer in Bragg-Brentano geometry, with a $\text{CuK}\alpha$ filtered beam ($\lambda = 0.15406 \text{ \AA}$) produced at 40 kV and 40 mA. The scanning range (2θ) was performed from 25° to 45° with a step size of 0.025° 2θ /s. XRD was used to assess the phases present, crystallinity of the powders as well as its crystallographic properties. Phase identification was performed with reference to the library database supplied by the International Centre for Diffraction Data (ICDD) by directly comparing the X-ray diffraction patterns to the Joint Committee for Powder Diffraction Standards (JCPDS) files for Aragonite (JCPDS, Card No. 24-0025). The crystallite sizes, in six reflection planes, were calculated based on the extraction of information from the X-ray diffraction patterns. Based on the Scherrer formula, the crystallite size can be calculated from the XRD peak breadth: $D = 0.9\lambda \text{ FWHM} \cos\theta$, where D is the crystallite size (nm), λ the wavelength of the monochromatic X-ray beam (0.15406 nm for $\text{Cu K}\alpha$ radiation), FWHM is the full width at the half maximum of the diffraction peak under consideration (rad), and θ the diffraction angle [$^\circ$]. Crystallinity is

a complex concept integrating the effects on the XRD of the crystal strain, diffraction domain size and crystal defects. The crystallinity percentage (X_c) was determined using the following equation:

$$X_c(\%) = \frac{\sum A_C}{\sum A_C + \sum A_A} \times 100$$

where $\sum A_C + \sum A_A$ gives the sum of the area under all the crystalline phases and amorphous peaks and $\sum A_C$ yields the sum of the areas under the crystalline peaks present in the scan range between 25 to 45°.

RESULTS

Measured shell parameters

The basic parameters (height, width and weight) of the *Cepaea* shells are presented in Table 1. The snails from this genus have medium-sized shells (height and width c.a. 15÷25 mm, weight c.a. 0.5 g).

TABLE 1. Shell parameters for genus *Cepaea*
TABELA 1. Parametry muszli ślimaków z rodzaju *Cepaea*

Shell character	<i>C. hortensis</i> (n = 30)	<i>C. nemoralis</i> (n = 30)
Height [mm]	15.25	18.44
min-max	14.12÷16.76	16.11÷20.64
Width [mm]	19.07	23.58
min-max	17.43÷21.72	21.54÷25.86
Weight [g]	0.48	0.79
min-max	0.34÷0.72	0.55÷1.2

Structures observed by SEM

The shells of *C. hortensis* and *C. nemoralis* have a crossed-lamellar structure. This structure is commonly observed in many representatives of Gastropoda, Bivalvia, Scaphopoda, and Polyplacophora [7]. The crossed-lamellar structure has already been described in many publications [7, 20-25]. This structure has been found in the shells of the following species from the Helicidae family: *Helix pomatia*, *Cornu aspersum*, *Arianta arbustorum*, *Caucasotachea vindobonensis* [21, 25]. The crossed-lamellar structure has a hierarchical character. This means that the shell is composed of several biomineral layers which are internally complex. The periostracum is the outermost layer of the shell. In the case of *C. hortensis* and *C. nemoralis* as well as other land snails, no internal division has been confirmed in the periostracum [7]. It is created before the biomineral layers and plays a role in the process of their formation [7, 26]. It is an organic matrix composed of proteins and polysaccharides, which provides stability to the calcium carbonate crystals and then to higher-order mineral structures [27, 28]. After shell formation, the periostracum creates a barrier that protects the deeper biomineral layers against the external environment [5]. The periostracum may undergo slow degrada-

tion as a result of, among others, mechanical abrasion and bioerosion [29, 30]. Below the periostracum, the structure of *C. hortensis* and *C. nemoralis* shells is composed of four biomineral layers made of first, second- and third-order lamellae of varied geometrical orientation, which is a typical feature for crossed-lamellar structures [21, 25] - Figures 1 and 2. The shape of the first-order lamellae is similar to a rectangular, and the lamellae are placed parallel to one another. They are made of second-order lamellae, which are composed of third-order lamellae. The second-order lamellae are shaped like sheets and they are aligned at an angle of 82° or 98° in relation to one another in two neighbouring first-order lamellae [21, 23, 31]. Third-order lamellae are shaped like rods. They have a different orientation in relation to one another in two neighbouring second-order lamellae [32]. The presence of four biomineral layers is considered characteristic for species with large shells like *Helix pomatia*, *Arianta arbustorum*, *Caucasotachea vindobonensis*. These layers, together with lamellae of varied orientation, reinforce the structure and at the same time, reduce the weight of the shell and limit the amount of calcium carbonate necessary for its formation [25].

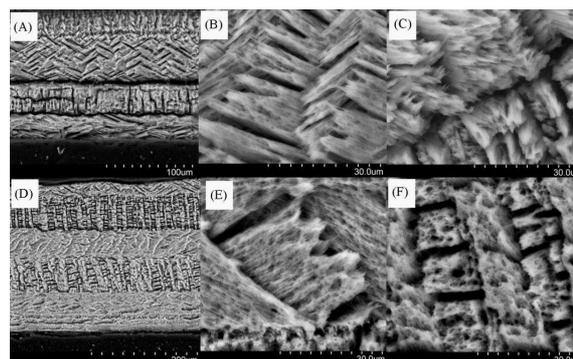


Fig. 1. SEM images of crossed lamellar structures occurring in (A-C) *Cepaea hortensis*, (D-F) *Cepaea nemoralis*. (A, D) Polished and etched sections with several layers with various orientations of first order lamellae. (B, E) Polished and etched sections showing second order lamellae and (C, F) third order lamellae

Rys. 1. Obrazy SEM struktury skrzyżowanych płytek występujących w (A-C) *Cepaea hortensis*, (D-F) *Cepaea nemoralis*. (A, D) Warstwy z widocznymi płytkami I rzędu o różnych orientacjach geometrycznych. (B, E) Warstwy z płytkami II i (C, F) III rzędu

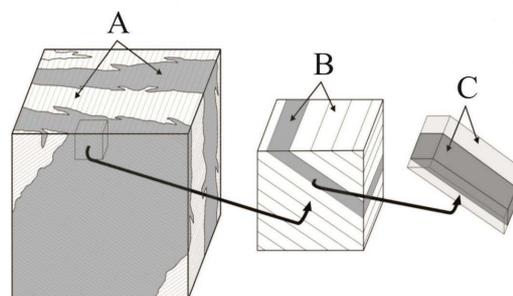


Fig. 2. Sketch showing crossed-lamellar microstructure and its constituent (A) first, (B) second and (C) third order lamellae units [12]

Rys. 2. Rysunek przedstawiający strukturę skrzyżowanych płytek z widocznymi jednostkami w postaci (A) I-, (B) II- i (C) III-rzędowych płytek [12]

Chemical analysis

A quantitative analysis of the chemical composition of the *Cepaea* shell (Table 2) showed a high content of Ca, O, and C, indicating the presence of well-crystallized calcium carbonate. Besides elements typical of calcium carbonate, traces of elements such as Si, Al, Mg, Na, P, S, Mn, Fe, Zn, and Sr were additionally identified. The presence of these elements, particularly those forming ions⁺², is related to the presence of impurities in the crystal lattice of calcium carbonate.

TABLE 2. Mean content of studied elements in *Cepaea hortensis* and *Cepaea nemoralis* shells

TABELA 2. Skład chemiczny muszli ślimaków *Cepaea hortensis* i *Cepaea nemoralis*

Elements	Chemical compositions [% weight]	
	<i>C. hortensis</i> (<i>n</i> = 5 [*])	<i>C. nemoralis</i> (<i>n</i> = 5 [*])
C	11.46	11.15
Na	0.05	0.03
Mg	0.03	0.04
Al	0.04	0.12
Si	0.77	0.66
P	0.09	0.07
S	0.22	0.06
Ca	39.74	39.96
Mn	0.03	0.04
Fe	0.08	0.03
Zn	0.18	0.11
Sr	0.15	0.22
O	47.16	47.51
Total	100	100

*n - number of polished sections each with 32 measurements

The admixtures in the shells of molluscs include mainly elements such as Sr, Mg, Fe, and Mn, which form divalent ions. The ions of these elements are capable of replacing divalent Ca ions in the crystal lattice of calcium carbonate. Theoretically, it is assumed that the ions of elements whose diameter is greater than Ca (e.g. Sr) penetrate the aragonite lattice more frequently, whereas elements with an ion diameter smaller than Ca (e.g. Mg, Fe, Mn) penetrate the calcite lattice. In the presence of carbonates, Sr - similar to the case of Ca - creates minerals (strontianite, aragonite) from the so-called aragonite group, which crystallize in the orthorhombic system. Meanwhile, the minerals of elements such as Mg, Fe, and Mn (magnesite, siderite, rhodochrosite) belong to the so-called calcite group and crystallize in the trigonal system [2, 33-35]. The minerals mentioned above (strontianite, magnesite, siderite, rhodochrosite), which do not contain Ca in their structure, have not been found in the shells of molluscs so far, however, the elements forming them may be present in the crystal lattice of calcium carbonate. The admixtures may also include Si and Al ions, which

affect shell growth, as well as Na, Mg, Zn, S and P that are found in among others, enzymes, body fluids and their derivatives [35-42].

X-ray analysis

The qualitative phase analysis indicates that calcium carbonate in the shells of *C. hortensis* and *C. nemoralis* is present in the form of aragonite. It is most often present in two crystallographic forms in the shells of molluscs, i.e. aragonite in the orthorhombic system and/or calcite in the trigonal system [35]. Amongst the representatives of the Helicidae family, aragonite has been confirmed in the structure of the shells of *Cornu aspersum* [43] and *Caucasotachea vindobonensis* (unpublished data). The peaks in Table 3 were used to determine the crystallite size. The average crystallite size in six reflection planes (*hkl* and positions) for all the shells was almost comparable - 34.39 nm for *C. hortensis*, 38.14 nm for *C. nemoralis*. For comparison, the average size of crystallites in the shells of *Caucasotachea vindobonensis* was 41.3 nm (unpublished data).

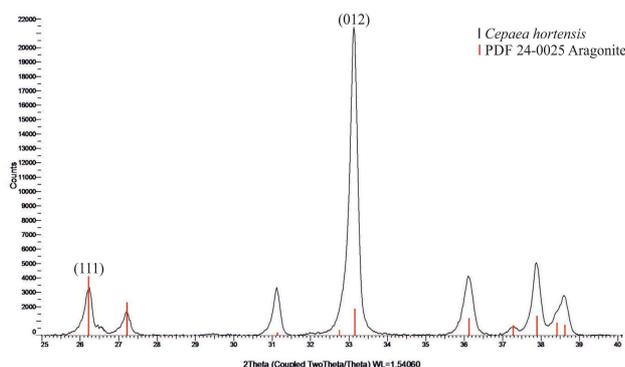
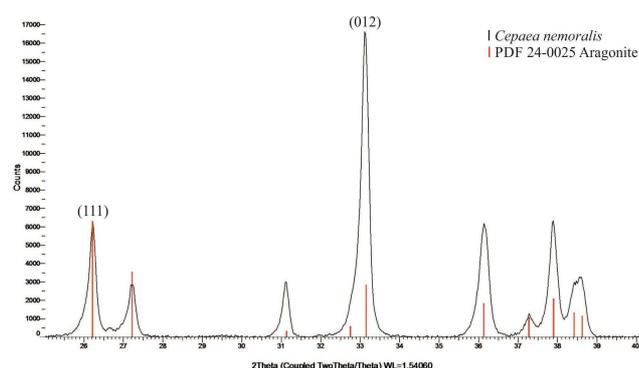
TABLE 3. Crystallite size of *Cepaea* shells

TABELA 3. Rozmiar krystalitów muszli ślimaków z rodzaju *Cepaea*

Peak position 2 θ	(<i>hkl</i>)	<i>C. hortensis</i> [nm] (<i>n</i> = 5)	<i>C. nemoralis</i> [nm] (<i>n</i> = 5)
26.213	(111)	35.54	42.57
27.216	(021)	38.70	43.34
31.116	(002)	39.16	43.24
33.128	(012)	29.08	32.34
45.853	(221)	33.30	35.27
50.229	(132)	30.54	35.12
Average		34.39	38.14

The strongest reflexes on the diffraction patterns from the *C. hortensis* and *C. nemoralis* samples have been assigned to planes (012) and (111). Theoretically, the (111) reflex is the strongest in the orthorhombic system. Reflex (012) is the strongest in the case of the *C. hortensis* and *C. nemoralis* shell samples (Figs. 3 and 4). Similar results were observed for the samples of *Caucasotachea vindobonensis* shells (authors' own unpublished data). It should be remembered that the crystallite size is not always equal to the particle size. In addition, most grains can be composed of crystalline domains and therefore, sometimes the crystallite size is smaller than the size of the grains.

Table 4 contains global values and a reduced area for the *Cepaea* genus patterns as well as the percentage value of the crystallinity of the investigated powders. The *C. hortensis* shell is the one with the most amorphous phase present in the structure - about 4.5%, while *C. nemoralis* contains 2.2%.

Fig. 3. X-ray diffraction patterns of *Cepaea hortensis* shell powderRys. 3. Dyfraktometr sproszkowanej muszli ślimaka *Cepaea hortensis*Fig. 4. X-ray diffraction patterns of *Cepaea nemoralis* shell powderRys. 4. Dyfraktometr sproszkowanej muszli ślimaka *Cepaea nemoralis*TABLE 4. Crystallinity [%] of *Cepaea* shellsTABELA 4. Udział fazy krystalicznej w [%] w muszlach ślimaków z rodzaju *Cepaea*

	<i>C. hortensis</i> [n = 5]	<i>C. nemoralis</i> [n = 5]
Global area	64.71	75.25
Reduced area	61.78	73.6
Crystallinity [%]	95.5	97.8

CONCLUSIONS

These are the first studies to compare the structure, basic crystalline structure and chemical composition of the shells of two taxons of the genus *Cepaea*. According to our results, the *Cepaea* snail shell is characterized by an orthorhombic aragonite fine crystalline structure. It has been found that the shell has a typical crossed-lamellar structure. There are visible differences in the number of some elements, depending on the species of snail. These differences are probably related to the environmental conditions in which the species lives. The study determined that the average crystallite size of the building layers of the shells does not differ significantly, however, the smallest crystallites are characteristic of *C. hortensis*, which also has the smallest of the two shells. It should also be noted that the crystallinity of the coating within the tested shells varies and the content of the amorphous phase or the organic matrix is

the smallest in the case of the *C. nemoralis* shells. Further work on the structure and physico-mechanical properties of *Cepaea* snails will be conducted.

The presented research complements knowledge and introduces new data on the structural, phase and chemical construction of *C. hortensis* and *C. nemoralis* shells. The article elaborates a research methodology which will be used in the works on the impact of the environmental conditions in which snails live on the development and structure of the shells, and as a result, their mechanical properties.

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