Chapter 8
Spectroscopic Investigation of Shell Pigments from the Family Neritidae (Mollusca: Gastropoda)

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Abstract Molluscan shells display a wide variety of pigmentation patterns. The diversity in molluscan shell color reflects the variety of different chemical species in the shell surface. Chemical characteristics of molluscan shell pigments have been extensively investigated, and compounds including porphyrins, polyenes, and melanins were identified as shell pigments. Here, we investigated shell pigments in 24 species in the family Neritidae using Raman spectroscopy. An excitation wavelength of 514.5 nm revealed two types of Raman spectra. One was characterized by two peaks ranging in wavenumber from 1100–1200 to 1500–1600 cm^{-1}, which indicate the presence of polyenes. Another type remained unassigned, implying the presence of other pigments such as porphyrins or melanins. The Raman spectra indicated a different distribution of the two types of pigments in shells. The patterns of the Raman spectra had no obvious relationship with taxonomical classification lower than the genus level and typical habitats. Measurement of the Raman spectrum at an excitation wavelength of 442 nm suggested that the wavelength can distinguish polyenes from other types of pigments.

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8.1 Introduction

The phylum Mollusca is the second largest taxon in the animal kingdom. It is very diverse in size, shape, color, habitats, and other traits. The diverse pigmentation patterns on shells are a key trait. Diversity in molluscan shell color reflects the wide variety of chemical species included in the shell. Investigating the origin of shell pigmentation is very important to understand the evolutionary history of Mollusca (Williams 2017). Furthermore, chemical speciation of shell pigment molecules could provide insight into the interaction of pigment molecules with other shell components (Hedegaard et al. 2006), which is important for the better understanding of biomineralization during shell formation.

Shell pigments have been extensively investigated (Williams 2017; Ishikawa et al. 2013 and references therein). Comfort (1949a, b) reported the presence of porphyrin compounds in several groups of gastropods including the family Neritidae (described later) using ultraviolet fluorescence and absorption chromatography. Subsequently, the presence of polyene compounds was reported using Raman spectroscopy. For example, Hedegaard et al. (2006) estimated the chain length of conjugated polyenes and discussed the chemical modification of polyenes contained in the shell of the gastropod *Cypraea moneta* (Cypraeidae). Polyene pigments were observed in various taxa. However, the target samples used in previous studies were limited to common shells with strong colors. Here, we comprehensively studied the family Neritidae and conducted a preliminary study to detect porphyrin pigments using Raman spectroscopy. The family Neritidae comprises small- or medium-sized snails that inhabit a wide variety of shallow-water environments such as brackish areas and intertidal rocky zones. The family is characterized by clear and vivid color bands (Fig. 8.1), which make spectroscopic analysis easy to perform. Their various habitats are expected to have an effect on pigmentation because shell colors are affected by environmental factors (e.g., Sokolova and Berger 2000).

8.2 Materials and Methods

We investigated 24 species from 5 genera of the family Neritidae by using Raman spectroscopy (Table 8.1). Raman spectra were obtained from each differently colored area using an exposure time of 10 s for each measurement. Ten spectra were obtained for each area. Estimated energy resolution ranged from 1 to 2 cm^{-1}. Two Raman spectrometers with different excitation wavelengths (514.5 and 442 nm) were used (Table 8.2). The excitation wavelength of 514.5 nm has been previously demonstrated to detect polyenes. The excitation wavelength of 442 nm is near the Soret maxima of porphyrins and thus is expected to excite porphyrins that are

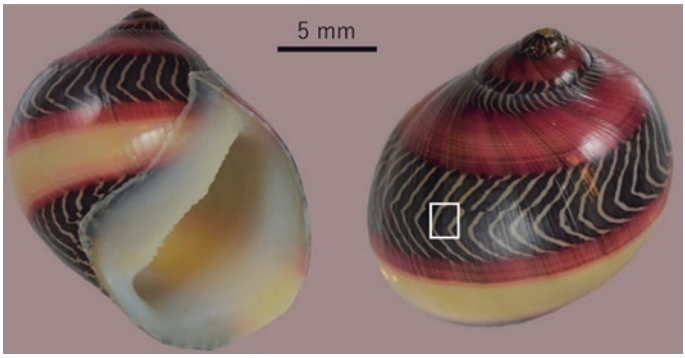

Fig. 8.1 *Neritina waigiensis* in the family Neritidae. A section of the shell denoted by the white square was used for Raman analysis (see Fig. 8.3)

Table 8.1 Collection numbers, species, locality, and typical habitats of the investigated samples

Coll. number	Species	Locality	Typical habitat
RM 32906	*Clithon oualaniense* (Lesson, 1831)	Bohol Island, Philippines	Brackish, on sandy or muddy substrate
RM 32907	*Clithon (Pictoneritina) chlorostoma* (Broderip, 1833)	Ishigaki Island, Japan	Under rocks, in estuaries
RM 32908	*Clithon cryptum* (Eichhorst, 2016)	Amami Island, Japan	Under rocks, in estuaries
RM 32909	*Nerita (Amphinerita) insculpta* (Récluz, 1841)	Okinawa Island, Japan	Intertidal, on rocks
RM 32910	*Nerita (Cymostyla) tristis* (Pilsbry, 1901)	Amami Oshima Island, Japan	Intertidal, on rocks
RM 32911	*Nerita (Cymostyla) striata* (Burrow, 1815)	Okinawa Island, Japan	Intertidal, on rocks
RM 32912	*Nerita (Ritena) plicata* (Linnaeus, 1758)	Ikema Island, Japan	Intertidal, on rocks
RM 32913	*Nerita (Ritena) costata* (Gmelin, 1791)	Amami Oshima Island, Japan	Intertidal, on rocks
RM 32914	*Nerita (Theliostyla) picea* (Récluz, 1841)	Australia	Uncertain
RM 32915	*Nerita (Theliostyla) exuvia* (Linnaeus, 1758)	Cebu Island, Philippines	Intertidal, on rocks
RM 32916	*Nerita (Theliostyls) albicala* (Linnaeus, 1758)	Amami Island, Japan	Intertidal, on rocks
RM 32917	*Nerita (Argonerita) signata* (Lamarck, 1822)	Bohol Island, Philippines	Intertidal, on rocks
RM 32918	*Nerita (Argonerita) chammaeleon* (Linnaeus, 1758)	Ishigaki Island, Japan	Intertidal, on rocks
RM 32919	*Nerita (Linnerita) incerta* (von dem Busch, 1844)	Zamami Island, Japan	Intertidal, on rocks

(continued)

Table 8.1 (continued)

Coll. number	Species	Locality	Typical habitat
RM 32920	*Neritina (Linnerita) rumphi* (Récluz, 1841)	Malakal Island, Palau	Intertidal, on rocks
RM 32921	*Neritina (Linnerita) polita* (Linnaeus, 1758)	Okinawa Island, Japan	Intertidal, on rocks near sand
RM 32922	*Neritina (Neritina) pulligera* (Linnaeus, 1767)	Okinawa Island, Japan	On rocks in rivers
RM 32923	*Neritina (Vittina) parallela* (Röding, 1798)	Cebu Island, Philippines	Brackish area
RM 32924	*Neritina (Vittina) waigiensis* (Lesson, 1831)	Cebu Island, Philippines	Mangrove swamp
RM 32925	*Neritina (Vittina) turritta* (Gmelin, 1791)	Cebu Island, Philippines	On mud in mangrove swamp
RM 32926	*Neripteron (Dostia) cornucopia* (Benson, 1836)	Cebu Island, Philippines	Brackish area and mangrove swamp
RM 32927	*Smaragdia rangiana* (Récluz, 1842)	Balicasag Island, Philippines	On seagrasses
RM 32928	*Neritodryas dubia* (Gmelin, 1791)	Cebu Island, Philippines	Intertidal, brackish area
RM 32929	*Neritodryas* sp.	Cebu Island, Philippines	Intertidal, brackish area

Scientific names and typical habitats follow the designation schemes of Tsuchiya and Kano (2017) and Eichhorst (2016)

Table 8.2 Excitation wavelength, laser source, laser power, calibration standard, and objective lens magnification data of the two Raman spectrometers

Excitation wavelength (nm)	Laser source	Laser power (mW)	Calibration standard	Objective lens magnification
514.5	Ar^+ laser	30	Naphthalene	×20
442	He-Cd laser	120	Silicon	×20

Laser power could be attenuated through the optical paths for both instruments

present in the mollusc shell (Comfort 1949a). We performed intact shell surface analysis without chemical treatments. The samples examined were registered in The University Museum, The University of Tokyo.

8.3 Results and Discussion

8.3.1 Raman Spectra at 514.5 nm

Figure 8.2 shows representative Raman spectra obtained from yellow, red, and black color bands of *N. waigiensis*. The Raman spectra of the black and red parts were similar, while the spectrum of the yellow part was markedly different from both. The spectrum obtained from the yellow part was characterized by two peaks with wavenumbers ranging from 1100–1200 cm^{-1} (ν_5) to 1500–1600 cm^{-1} (ν_1). These two peaks are assigned to polyene pigments contained in the shell (e.g., Bergamonti et al. 2013). These polyene-type spectra were obtained from the species listed in Table 8.3. While Raman shifts of ν_5 and ν_1 varied in all species, the observed values were within a certain range.

The spectra obtained from red and black parts of *N. waigiensis* had no obvious peaks that could be assigned to polyenes. This type of spectrum was also obtained from the species listed in Table 8.4. Similar spectra from the gastropod *Clunculus pharaonius* (Trochidae) were reported by Merlin and Delé-Dubois (1986) and Williams et al. (2016). The red and black pigments of *C. pharaonius* were identified as uroporphyrin and eumelanin, respectively, using high-performance liquid chromatography (Williams et al. 2016). However, the authors argued that the Raman spectra did not convey any definitive information concerning shell pigments, and it

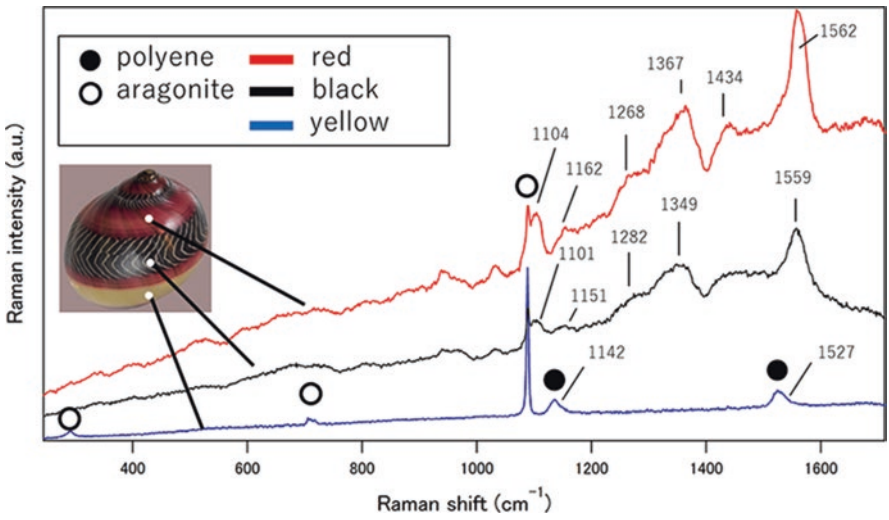

Fig. 8.2 Raman spectra obtained from each color band of *N. waigiensis* using 514.5 nm excitation. White circles in the figure indicate measurement points for Raman analysis

Table 8.3 Colors and wavenumbers of the ν_5 and ν_1 peaks using the 514.5 nm excitation from species containing polyenes

Species	Color	ν_5 (cm^{-1})	ν_1 (cm^{-1})
Clithonoualaniense (Lesson, 1831)	Pale yellow	1139	1532
Nerita (Amphinerita) insculpta (Récluz, 1841)	Black	1138	1528
Nerita (Linnerita) incerta (von dem Busch, 1844)	Green	1138	1526
Nerita (Cymostyla) tristis (Pilsbry, 1901)	Deep green	1138	1530
Nerita (Cymostyla) striata (Burrow, 1815)	Black	1135	1525
Nerita (Cymostyla) striata (Burrow, 1815)	Light green	1136	1527
Nerita (Ritena) plicata (Linnaeus, 1758)	Black	1135	1524
Nerita (Argonerita) signata (Lamarck, 1822)	Red	1133	1523
Nerita (Argonerita) chammaeleon (Linnaeus, 1758)	Black	1138	1524
Nerita (Theliostyls) albicilla (Linnaeus, 1758)	Deep green	1137	1527
Neritina (Linnerita) rumphii (Récluz, 1841)	Black	1136	1526
Neritina (Linnerita) rumphii (Récluz, 1841)	Green	1137	1525
Neritina (Linnerita) polita (Linnaeus, 1758)	Deep green	1135	1524
Neritina (Linnerita) polita (Linnaeus, 1758)	Green	1135	1524
Neritina (Neritina) pulligera (Linnaeus, 1767)	Deep green	1138	1528
Neritina (Neritina) pulligera (Linnaeus, 1767)	Orange	1139	1530
Neritina (Vittina) waigiensis (Lesson, 1831)	Yellow	1142	1527
Smaragdia rangiana (Récluz, 1842)	Green	1139	1534
Nerita (Ritena) costata (Gmelin, 1791)	Black (between spiral ribs)	1134	1524

was quite unclear whether the obtained spectra were assignable to uroporphyrin or eumelanin included in the shell. Presently, we also could not conclusively identify shell pigment from the Raman spectra. However, the difference in chemical origins of the black and red pigmentations of *N. waigiensis* from that of yellow pigmentation is a novel finding. It is worth noting that the Raman peaks obtained from red and black regions of *N. waigiensis* appeared at a similar frequency to those in the spectrum of eumelanin reported by Mbonyiryivuze et al. (2015). It is conceivable that the peaks we observed were due to background fluorescence.

Shell pigmentation may be relevant to taxonomical classification (Comfort 1949a, Williams 2017). However, the patterns and peak wavenumber of the Raman spectra (Tables 8.1, 8.2, and 8.3) had no obvious relationship with the taxonomical classification at the genus or lower level or with typical habitats. Different types of spectra could be observed even in the same individuals.

We fabricated the shell section perpendicular to the apertural margin of *N. waigiensis* (Fig. 8.3). The thickness of red- and black-colored layers was approximately 30 μm. The Raman spectra obtained from five points are shown in Fig. 8.4. No obvious peaks assignable to polyenes were observed from points 1 to 2. Two weak peaks assignable to polyenes were detected at point 3. The obtained spectra

Table 8.4 Colors and Raman shifts of representative peaks obtained at 514.5 nm excitation wavelength from species displaying other spectra

Species	Color	Raman shifts of representative peak (cm^{-1})
Clithon oualaniense (Lesson, 1831)	Black	1562
Clithon (Pictoneritina) chlorostoma (Broderip, 1833)	Black	1562
Clithon cryptum (Eichhorst, 2016)	Black	1566
Nerita (Linnerita) incerta (von dem Busch, 1844)	Deep green	1562
Nerita (Theliostyla) picea (Récluz, 1841)	Black	1560
Nerita (Theliostyla) exuvia (Linnaeus, 1758)	Black	1548
Neripteron (Dostia) cornucopia (Benson, 1836)	Deep green	1559
Neritina (Vittina) waigiensis (Lesson, 1831)	Red	1562
Neritina (Vittina) waigiensis (Lesson, 1831)	Black	1557
Neritina (Vittina) turritta (Gmelin, 1791)	Black	1566
Neritina (Vittina) parallella (Röding, 1798)	Black	1558
Neritodryas dubia (Gmelin, 1791)	Deep purple	1560
Neritodryas sp.	Red	1560
Neritodryas sp.	Black	1565
Nerita (Ritena) costata (Gmelin, 1791)	Black (on spiral ribs)	1554

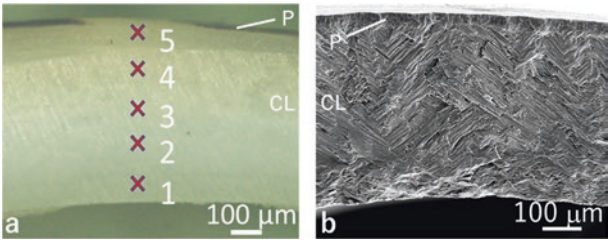

Fig. 8.3 (**a**) A section perpendicular to the apertural margin of *N. waigiensis*. The black and yellow patterns on the shell exterior are visible on the upper side of the figure. The numbers 1–5 indicate measured points. (**b**) Scanning electron microscopy image of the section of *N. waigiensis*. The multilayered structure of the shell is evident. The outermost region is a prismatic layer (P) that corresponds to the colored layer in (**a**). The inner layer has a crossed-lamellar structure (CL). These two figures were obtained from different individuals of *N. waigiensis*

raise the possibility that the concentration of polyenes increases continuously from the interior of the shell to the exterior surface. In contrast, black and red pigmentation appeared only in the narrow range on the surface, indicating that their distributions are quite different from the distribution of polyenes.

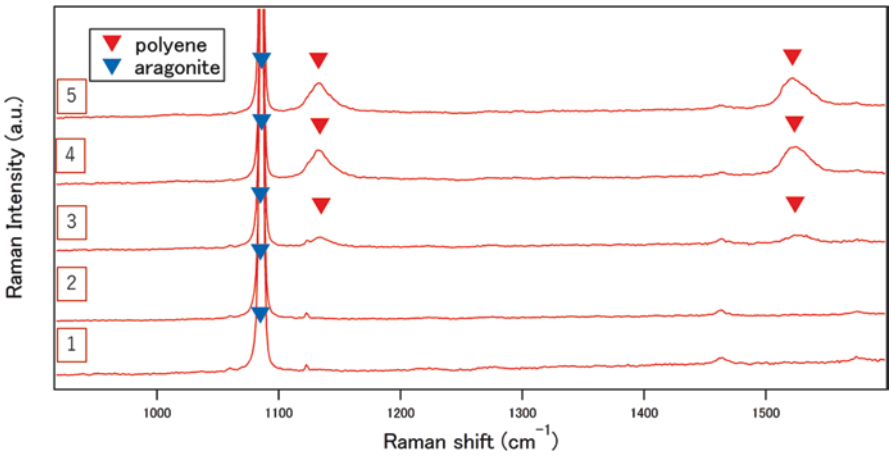

Fig. 8.4 Raman spectrum obtained from each measured point in Fig. 8.3

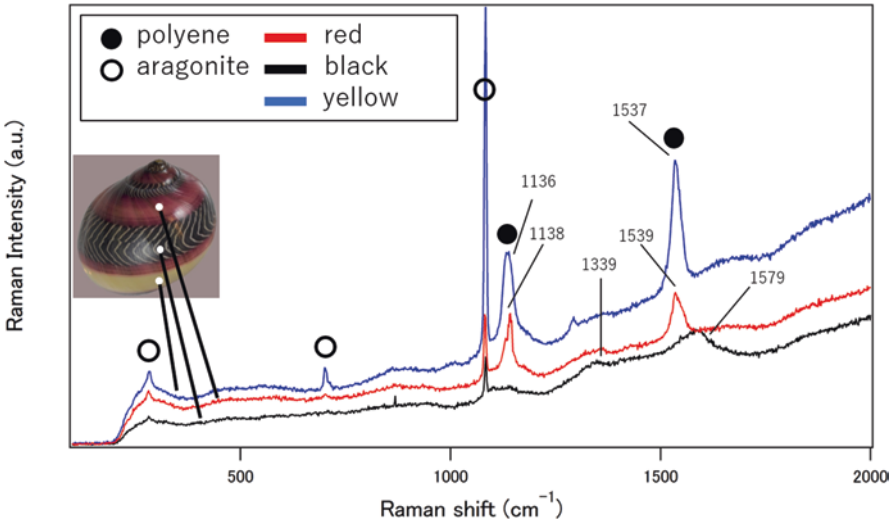

Fig. 8.5 Raman spectrum obtained from each color band of *N. waigiensis* using the 442 nm excitation wavelength. The white circles in the figure indicate the measurement points for Raman analysis

8.3.2 Raman Spectra at 442 nm Excitation

Figure 8.5 shows Raman spectra obtained from *N. waigiensis* using the 442 nm excitation wavelength. Clear peaks assignable to polyenes were observed from the yellow and red parts of the shell surface, but not from the black part. Polyene-type

spectrum from the red part may be attributed to the polyenes of the yellow part existing just under the red color band on the surface. Williams et al. (2016) argued that it is difficult to distinguish polyenes from porphyrins only by the Raman spectra. However, our results suggest that polyenes can be distinguished from other pigments because the background fluorescence was relatively low compared to that for the peak heights at ν_5 and ν_1.

8.4 Conclusions

Analysis using the 514.5 nm excitation wavelength demonstrated that the pigmentation in the family Neritidae has at least two types of origins: polyenes and other unknown pigments such as porphyrins or melanins. The data from a shell section of *N. waigiensis* suggest that these two types of pigments are distributed in a different manner in the section. The patterns of the Raman spectra did not display obvious relationships with taxonomical classification and habitats. The Raman spectra obtained using the 442 nm excitation wavelength suggest that polyenes can be distinguished from other pigments by the presence of strong peaks of ν_5 and ν_1. However, definitive information on shell pigments was difficult using only Raman spectroscopy. Other analytical techniques such as high-performance liquid chromatography could be needed and will be studied.

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