Mechanics of Cocoon Secretion in a Segmented Worm
(Annelida: Hirudinidae)

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Abstract

Clitellate annelids (e.g., segmented earthworms, leeches) secrete proteinaceous cocoons into which eggs are deposited. The process of cocoon production is characterized by the coordinated release of micro-granules from secretory cells positioned asymmetrically within the clitellum. Collectively, these assemble into a tubular cocoon sheath that is sealed at either end by globular opercula. By transmission electron microscopy (TEM), we show here that granules destined to the cocoon operculum in the leech, *Erpobdella obscura*, display a series of concentric rings surrounding a structureless core with dimensions approximating a single nanoglobule found in the operculum. Upon their channeling to the surface through narrow tubules, granules are secreted into the cocoon lumen where they appear to fragment upon contact with the operculum matrix. The distribution of partial concentric ring structures throughout the operculum suggests that granular fusion causes dynamic fragmentation of outer surface material, which thereafter integrates into operculum nanoglobules and cavities. Other granules within the same secretory cell display a punctate pattern and likely fuse with the cocoon sheath prior to crystallization.

Keywords: clitellate, reproduction, biomaterial, egg case, operculum, granule

1. Introduction

Clitellate annelids (e.g., earthworms, leeches) secrete egg sacs, or cocoons, from specialized midbody segments known as the clitellum. This process is characterized by formation of a cocoon sheath/wall that initially surrounds the clitellum and into which nutritive cocoon fluid and eggs are deposited. Thereafter, the cocoon sheath is passed over the worm's head while it is concomitantly sealed at both ends by glue-like plugs, called opercula (Hirao 1965; Wilkialis 1975). In a few leech species (e.g., *Haementeria ghilianii*; *Helobdella papillornata*), cocoons are secreted as a sac encircling the ventral gonopores, and are sealed with a single operculum. The completed cocoon is deposited onto the substratum (or sometimes on the venter of leeches) where it serves as protective barrier and microenvironment for embryonic development.

Clitellate worms secrete various cocoon types depending on constraints imposed by environmental pressures and developmental strategies (e.g., oviparous vs. albuminotrophy; Sawyer 1984). For example, mechanically strong, hard-shelled cocoons are abandoned by the parent worm, leaving embryos to develop independently on nutritive cocoon fluid (albuminotrophy), while large, yolky (oviparous) eggs are typically deposited into membranous or gelatinous cocoons that are often brooded by parent leeches (Davies and Oosthuizen 1992; Govedich 2004; Siddall et al. 2005). In an unusual display of parental care, species of *Theromyzon* cement membranous cocoons to their venter, and upon hatching bring juveniles to their first blood meal, a duck nare (Wilkialis and Davies 1980).

Despite very different cocoon types that accommodate various life cycle requirements, all Clitellata appear to build cocoons by the coordinated release of proteinaceous micro-granules that are synthesized within the clitellum. The process of granule synthesis and secretion has been examined in a few clitellate species (e.g., *Branchiobdella pentodonta*, *Eisenia foetida*, *Enchytraeus fragmentosus*, *Lumbricillus rivalis*, *Tubifex hattaï*), leading to the identification of distinct granule types that contribute material to the cocoon wall and opercula, respectively (Hirao 1965; Farnesi and Vagnetti 1973; Hess and Vena 1974;

Richards 1977; Suzutani-Shiota 1980; Morris 1983). The ultrastructure of the cocoon wall is characterized by stacked, fibrous layers that, when sectioned obliquely, display Bouligand-like patterns with varying step-angles (Knight and Hunt 1974; Dimitriu and Shain 2004; Marotta et al. 2007). In contrast, opercula display random, porous arrays of spherical nanoglobules with no obvious ultrastructural organization (Rossi et al. 2013). Fully constructed cocoon wall and operculum biomaterials display extraordinary physical properties, including mechanical strength and thermal/chemical resilience (Mason et al. 2004; Coleman et al. 2008).

The process by which clitellar micro-granules segregate and assemble into the cocoon wall and operculum, respectively, remains enigmatic. Clearly, the material components of the cocoon are contained within granules, but intermediate steps progressing from granule synthesis to the apparent fusion of granules and self-assembly of cocoon structures, remains mostly unexplored. Here, we approached this question by sectioning through specimens of the leech, *Erpobdella obscura*, in the process of secreting a hard-shelled cocoon. An unexpected morphological structure, namely electron dense concentric rings, targeted a set of granules as operculum-bound. The scattered remnants of these structures suggest that outer layers of the granule fragment upon contact with existing cocoon material and integrate into the operculum matrix, while the inner core is released as a single nanoglobular particle.

2. Materials and Methods

2.1. Specimens

Erpobdella obscura specimens were collected from Haddon Lake, NJ, and *Theromyzon tessulatum* specimens were from Golden Gate Park, CA. Individuals were maintained in glass bowls in 0.3% Instant Ocean, as described (Mason et al. 2005).

2.2. Histology

Specimens were processed for histological sections according to Sayers et al. (2009). Briefly, leeches were fixed in 10% formaldehyde, dehydrated in an ascending ethanol series to xylene, embedded in Paraplast Extra[®] and sectioned at 3-4 μm . Sections mounted on glass slides were stained with Masson's trichrome (Sheehan and Hrapchak 1980). Images were captured with a Canon Rebel XT camera coupled with a Zeis Universal microscope using a PI 232 mm 28 adaptor (Perspective Image LLC, Beaverton, OR).

2.3. Scanning Electron Microscopy

Cocoons were fixed in 70% ethanol and selected under a stereomicroscope for visible and protruding opercula. After ethanol series dehydration, cocoons were critically-point dried with CO₂ before manual fracturing into equal halves or complete operculum removal to expose interior operculum and cocoon wall surface ultrastructure. Cocoon pieces were then mounted with carbon tape on aluminum studs, sputter coated with gold/palladium and imaged with a LEO1450EP scanning electron microscope.

2.4. Transmission Electron Microscopy

Specimens were fixed overnight in a buffered aldehyde fixative (2.5% paraformaldehyde, 2% glutaraldehyde, 0.1M sodium cacodylate, pH 7.2) for 2 h at 4°C, washed in 0.1M sodium cacodylate buffer (pH 7.2) and post fixed in 1% osmium tetroxide. To enhance image contrast, tissues were pre-stained with 2% uranyl acetate for 2 h at 4°C prior to ethanol dehydration and propylene oxide solvent transition. After solvent infiltration with ascending concentrations of Spurr's resin, tissue was embedded in pure Spurr's resin for 4 h at room temperature and then cured in plastic molds with fresh Spurr's resin at 60°C for 48 h. Ultrathin sections were made with an LKB ultramicrotome fitted with a diamond knife, collected on 100 and 200 mesh grids, and viewed in a Zeiss EM 902 electron microscope at 50 kV equipped with electron energy loss spectroscopy (EELS).

3. Results

A fully assembled, hard-shelled cocoon secreted by the leech, *Erpobdella obscura* is shown in Figure 1. Histological cross-sections within the clitellum of a mature parent leech in the process of egg laying revealed the presence of abundant granule-secreting cells with associated, granule-filled tubules (Fig. 2). To examine ultrastructural features within the cocoon-associated micro-granules targeted for secretion, we cut through corresponding clitellar segments in cross-section and examined by TEM. Sacs of granules were identified in the cell body that displayed a series of electron dense, concentric rings (electron dense lamellae—EDL) surrounding a structureless core; within the same region, other granules lacked rings and displayed distinct punctate patterns (Figs. 3, 4). Both types of granules were grouped in sacs containing 5-10 granules, respectively (Fig. 3). The relatively narrow width of tubules extending to the body surface appeared to restrict granule flow to 1-2 at a time (Fig. 5).

The release of granules into the cocoon lumen followed by their putative fusion with existing cocoon wall material and subsequent crystallization was captured in a different species, *Theromyzon tessulatum* (Fig. 6). The presumptive fusion of operculum-bound granules in *E. obscura* led to fragments of EDL appearing within, surrounding and spanning cavities of the operculum matrix (Fig. 7). These fragments acquired random shapes and contained variable layers, but their dimensions were consistent with EDL observed within granules of the cell body and tubules (*cf.* Figs. 4-7). Note that electron-dense shards (*e.g.*, Fig. 7) appear to be a component of the operculum matrix, as described previously (Rossi et al. 2013).

Fully formed operculum material was characterized by clusters of associated nanoglobules with dimensions ranging from 50-300 nm (Fig. 8; Rossi et al. 2013), while the ultrastructure of the cocoon sheath displayed fibrous and punctate (cross sections) patterns reminiscent of granules observed in the cell body (*cf.* Figs. 4B, 8B). Using approximate dimensions of granules and the assembled cocoon, we estimate that $1-2 \times 10^7$ granules of each type were required to construct a single *E. obscura* cocoon (cocoon sheath + opercula).

4. Discussion

Employing the leech, *Erpobdella obscura*, as a representative subject, we identified two distinct granule types within clitellar tissue that likely contribute material to the cocoon sheath/wall and opercula, respectively. The occurrence of both granule types proximal to each other (i.e., within the same cell body; see Fig. 3) was reported within Type C clitellar cells of *Tubifex hattai* (i.e., azocarmine- and aniline blue-staining, respectively; Hirao 1965), but differs from that observed in the leech *Theromyzon tessulatum*, in which cocoon wall- and operculum-bound granules are partitioned into regionally separated cell types (i.e., Type II/III cells; Sayers et al. 2009). Likewise, clitellar secretory cells in other species (e.g., in *Tubifex hattai*, *Enchytraeus fragmentosus*, *Lumbricuillus rivalis*) typically contain only one granule type and are asymmetrically positioned in the clitellum (e.g., Hess and Vena 1974; Richards 1977; Suzutani 1977; Suzutani-Shiota 1980). In contrast, clitellum granules identified in the giant Amazonian earthworm, *Rhinodrilus priolii*, appear to contain both cocoon wall and operculum material within the same granule (Lang et al. 2012). Thus, considerable variation in granular content appears to be tolerated across species types. Aside from some positional asymmetry within the clitellum, however, the mechanism by which cocoon wall or operculum material segregates to its structural target in any species remains unclear.

In *E. obscura*, a previously undescribed structure--namely a series of electron dense concentric rings (EDL)--marks these granules as operculum-bound based on EDL fragments found in the assembled operculum (cf. Figs. 3, 6). The central core of these granules comprise a structureless sphere with dimensions comparable with that of nanoglobules (~100-200 nm) that associate to form opercula (see Fig. 8A; Rossi et al. 2013). Thus each granule appears to contribute a single nanoglobule to the mature operculum, requiring $\sim 10^7$ granules in total. The randomly scattered fragments of EDL within operculum nanoglobules and cavities suggest that outer layers of operculum-bound granules fragment upon fusion with other granules (or operculum material), thus

releasing the internalized nanoglobule which thereafter associates with the operculum matrix. In some cases, EDL fragments associate permanently with free nanoglobules and become structural components of the operculum.

The composition of EDL remain unknown. Previous studies suggest that the cocoon wall and opercula are built from the same protein in *T. tessulatum* (Tcp; Mason et al. 2004); specifically, a glycosylated form of Tcp exists as a monomer and contributes to nanoglobules that comprise opercula, while de-glycosylated Tcp proteins assemble into polymers that characterize the fibrous cocoon wall (Mason et al. 2005; Sayers et al. 2009). Possibly, EDL in *E. obscura* represent polymeric fibers surrounding a glycosylated, structureless core, and upon fusion EDL fragments integrate into both opercula and the cocoon wall (the latter of which cannot be distinguished from other fibrous material). Indeed, EDLs display some resemblance to spiral “fingerprint” patterns in cocoon wall-bound granules of *T. hattai* (Suzutani 1977; Suzutani-Shiota 1980). Alternatively, EDL may be the remains of Golgi lamellae from which the structureless core (nanoglobule) is synthesized, though the energetic requirements for encircling all $\sim 10^7$ nanoglobules with Golgi layers would likely be prohibitive to operculum production.

Granules likely destined to the cocoon wall in *E. obscura* have a pocked appearance similar to that observed in *Branchiobdella pentodonta*, *Lumbricillus rivalis* and *Eisenia foetida*, which are thought to be in a paracrystalline state (Farnesi and Vagnetti 1973; Richards 1977; Morris 1983). The spatial organization of electron dense material within some of these *E. obscura* granules (e.g., see Fig. 4B) suggests an early stage of polymerization, since no distinct fibrous patterns were observed despite their random orientations in TEM sections (i.e., polymerized material would appear pocked in cross-section, but fibrous if rotated in any other direction). Regardless of the species, granular material is likely to be in paracrystalline form prior to fusion with existing cocoon wall material to facilitate its integration into the fibrous matrix. Indeed, polymerization and cocoon hardening appears to be delayed in *E. obscura* (and *T. tessulatum*;

see Fig. 6) until after granular fusion, which may allow time for the parent leech to physically shape the cocoon into its mature form (Brumpt 1900).

5. Conclusion

The detailed examination of related cocoon types (e.g., gelatinous, membranous, hard-shelled) in the context of granular content and progression through the secretory process will be necessary to resolve the different morphologies and strategies of cocoon production among diverse clitellates (and possibly their ancestral, tube-dwelling polychaetes). From analyses to date, however, it seems clear that the major structural components of the cocoon are built by the timely production and coordinated secretion of distinct micro-granule types (up to $\sim 4 \times 10^7$ granules in total per *E. obscura* cocoon), which then segregate and self-assemble into the fibrous cocoon wall and nanoglobular opercula.

Acknowledgments

Supported by NSF grant DBI-0216233 to DHS.

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Figure legends

Figure 1. Fully assembled cocoon from the leech, *Erpobdella obscura*. The cocoon wall (CW) forms a tube that is sealed at either end by opercula (arrows). Scale bar 500 μm . Insert shows an outwardly protruding operculum. Scale bar 100 μm .

Figure 2. Histological cross-sections through the clitellum of an *Erpobdella obscura* parent leech in the process of cocoon secretion. A) Clitellar tissue (CT) containing secretory granule cells was observed between the gut (g) and external cuticle (arrowhead). Scale bar 100 μm . B) Granule cells (Gc) were abundant within the CT. Scale bar 40 μm . C) Gc and tubules (Tu) were observed throughout the CT. Scale bar 10 μm . D) Granules were apparent within Gc and Tu. Scale bar 4 μm .

Figure 3. Granules within a clitellar secretory cell. Operculum-bound (star) and cocoon wall-bound (diamond) granules were in sacs (arrows) containing 5-10 granules per sac. Scale bar 100 nm.

Figure 4. Distinct granule types within the clitellum of *Erpobdella obscura*. A) Operculum-bound granules displayed electron dense lamellae (EDL) surrounding a structureless core with dimensions comparable with mature nanoglobules (Ng) found in opercula. B) Cocoon wall-bound granules (diamond) displayed organized, punctate patterns. Scale bars 100 nm.

Figure 5. Operculum-bound granules (star) in a narrow tubule. Between 1-2 granules were channeled to the epidermal surface by tubules. Adjacent cocoon wall-bound granules (diamond) are shown. Scale bar 100 nm.

Figure 6. Interactions of granules with existing cocoon material in *Theromyzon tessulatum*. A) Putative fusion of a granule with the cocoon wall; arrow identifies

the interface site. B) Regions of paracrystallization are adjacent to polymerized, fibrous material (arrows). Scale bars 100 nm.

Figure 7. Fragments of electron dense lamellae (EDL) integrated into the operculum matrix (OM). EDL fragments (arrows) partially filled (A, B) and/or spanned (B) cavities (c), lined cavities (A, B), appeared on surface OM (C) or were embedded with the OM (D). Scale bars 100 nm. Electron-dense shards observed in some panels have been described previously (Rossi et al. 2013).

Figure 8. Ultrastructure of major *Erpobdella obscura* cocoon components. A) Nanoglobules comprising operculum material. Arrow identifies a single nanoglobule; c – cavity. Scale bar 500 nm. B) Dashed line separates adjacent layers of polymerized fibrils in cross (upper) and oblique (lower) orientations within the crystallized cocoon wall. Scale bar 50 nm.

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