Ultrastructure and Sulfur Cytochemistry of Nematocyst Development in Catch Tentacles of the Sea Anemone \textit{Haliplanella luciae} (Cnidaria: Anthozoa)

GLEN M. WATSON and RICHARD N. MARISCAL

Department of Biological Science, Florida State University, Tallahassee, Florida 32306

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Nematocysts in catch tentacles of the sea anemone \textit{Haliplanella luciae} are formed by a Golgi-microtubule complex in which the microtubules appear to support the developing nematocyst during its formation. The newly formed capsule becomes wrinkled with the discontinuation of microtubule support, but becomes straight again as the nematocyst wall thickens (possibly from the addition of new material onto the wall surface). Final wall maturation is characterized by a decrease in wall thickness by a factor of two. Wall thinning is apparently caused by disulfide bond formation among wall molecules, because it can be reversed in the mature nematocyst by performic acid oxidation. The mature thread wall stains with Alcian blue both prior to and after performic acid treatment, whereas the mature capsule wall is stained only after such treatment. It is proposed that the thread wall has “free” sulfur groups that are absent in the capsule wall. © 1984 Academic Press, Inc.

The nematocyst or stinging organelle of cnidarians is a complex, intracellular structure containing a highly folded thread. Nematocyst discharge involves the forceful eversion of the thread through the capsule apex located at the cnidocyte plasma membrane. Eversion places the thread in contact with the prey or other target. Different nematocyst types have different functions ranging from adhesion to prey immobilization by means of toxin injection through the thread (see Mariscal, 1974, for a review).

Despite a diversity of function, many nematocysts are similar in chemical composition. Capsule and thread walls of various nematocyst types are known to be composed of a collagen-like protein crosslinked by disulfide bonds, because treatment of nematocysts with reducing agents such as dithiothreitol dissolves the capsule and thread (Blanquet and Lenhoff, 1966; Mariscal and Lenhoff, 1969; Fishman and Levy, 1967).

The presence of disulfides in the nematocyst capsule wall has also been demonstrated by performic acid treatment. Performic acid generates “free” sulfur groups by oxidizing disulfides to sulfonates (Blanquet and Lenhoff, 1966). The sulfonates are stained by Alcian blue dissolved in sulfuric acid (pH 0.2–0.3), which is specific for sulfur at a pH under 2.0 (Pearse, 1968), or under 1.0 (Hayat, 1975). Nematocysts that are not pretreated with performic acid, or that are placed in strong acid (e.g., formic acid) instead of performic acid are not stained by Alcian blue (Blanquet and Lenhoff, 1966). Performic acid treatment also causes the capsule wall to collapse or wrinkle, again due to the oxidation of disulfide bonds (Blanquet and Lenhoff, 1966). Recent studies employing X-ray microanalysis have also shown a high sulfur content in a variety of nematocysts (Mariscal, 1980, 1984).

Published electron microscopic studies on nematocyst development have shown that the capsule is formed by a Golgi apparatus associated with microtubules that surround the capsule in a cage-like array (Slautterback, 1963; Westfall, 1966; Carré, 1972;...

No study has yet attempted to reconcile the biochemical evidence on nematocyst composition (described above) with the ultrastructural observations on nematocyst development and nematocyst structure.

In the present study, using developing and mature nematocysts from catch tentacles of the sea anemone Haliplanella luciae, we present evidence suggesting that the Golgi-associated microtubules support the developing capsule wall as it forms, possibly from precursors in the nematocyst interior. We show that wall maturation is characterized by a decrease in wall thickness apparently caused by disulfide bond formation within the wall matrix. In addition, we present cytochemical evidence showing that the mature capsule wall differs chemically from the thread wall. Such chemical differences are discussed in terms of their implications for capsule and thread functioning.

MATERIALS AND METHODS

Induced Catch Tentacle Formation

Unlike sea anemone feeding tentacles, catch tentacles (specialized for aggression, Williams, 1975; Purcell, 1977) have a maturity gradient along the tentacle length such that mature nematocysts occur at the tentacle tip, while immature nematocysts occur at the tentacle base (Watson and Mariscal, 1983b). Because large numbers of developing nematocysts of a single type (classified as holotrichous isorhizas using Mariscal, 1974), and at a similar stage of development, are present at a given point along the tentacle length, catch tentacles are particularly suited for a study of nematocyst development.

Catch tentacles develop from feeding tentacles after nonclonemate sea anemones are placed together in crowded anemone cultures (Purcell, 1977; Watson and Mariscal, 1983a). For purposes of catch tentacle induction, specimens of H. luciae were collected along with specimens of the anemone Diadumene gracillima from separate oyster clumps near the Florida State University Marine Laboratory, Turkey Point, Florida. The anemones were crowded together in culture dishes filled with natural seawater (28-30‰), which was changed daily and held at 17-19°C. Newly developed catch tentacles (=stage 3 catch tentacles, Watson and Mariscal, 1983a) formed in the H. luciae after the first week. Such catch tentacles were removed from the anemones and processed for electron microscopy by methods described below.

Transmission Electron Microscopy

Specimens of H. luciae were anesthetized in their culture dishes using a one-to-one solution of 7.5% MgCl₂ and seawater. Catch tentacles were removed from the animals using fine scissors and drawn into 100-μl micropipets. The tentacles were dropped into 2.5% glutaraldehyde in Millipore-filtered (0.45-μm pore size) seawater (FSW) and fixed for 2 hr at room temperature. The tissue was washed in FSW and glass-distilled water (GDW), then placed in 1% OsO₄ and 0.15% ruthenium red (Polysciences, Inc.) in GDW for 3 hr at room temperature. Occasionally, tentacles were postfixed in 1% OsO₄ in GDW (i.e., without the ruthenium red). Tentacles were washed in GDW, dehydrated in a graded acetone series, and then embedded in Spurr Low-Viscosity Embedding Medium (Polysciences, Inc.). The material was sectioned using fresh glass knives and poststained with 2.0% uranyl acetate in 50% methanol for 5 min, followed by 0.2% lead citrate for 1 min. Sections were viewed using a Philips 201 transmission electron microscope. Measurements of structures were taken from micrographs using a 20 × measuring microscope (Ted Pella, Inc).

Sulfur Cytochemistry

The performic acid-Alcian blue staining procedure for demonstrating sulfur was described in the introduction. Previous attempts at using Alcian blue at the ultrastructural level have been somewhat unsuccessful because Alcian blue deposits are not particularly electron dense (see Hayat, 1975, for a complete discussion). In the present study, all attempts at en bloc staining following aldehyde fixation (using a variety of fixatives) were unsuccessful in tissues that were adequately preserved due to poor penetration of the stain. Therefore, a poststain regime was developed that allowed equal access of the staining medium to all cellular components. Glutaraldehyde-fixed tentacles were dehydrated in acetone and embedded in Spurr resin. Sections on copper grids were immersed for 8 hr in a 3% solution of Alcian blue 8GX (Sigma Chemical Co.) in 1 M sulfuric acid. The Alcian blue solution was heated to 70°C and filtered prior to use (after Pearse, 1968). Sections were then stained in a graded ethanol series. Alcian blue-stained sections were counterstained in uranyl acetate and lead citrate.
Discharged Nematocysts

Catch tentacles were removed from anesthetized H. luciae and placed in fresh seawater. Once the tentacles showed signs of recovery from the anesthetic (i.e., exhibited muscle contraction), they were drawn into a suction electrode and stimulated with a single pulse from a Grass S-5 Stimulator (Grass Instruments, Inc.). Electrical stimulation caused massive nematocyst discharge at the catch tentacle tip. The tentacles were drawn into micropipets and then dropped into 2.5% glutaraldehyde in FSW.

In discharged holotrich nematocysts, the thread everts to form a hollow tube. Hence, Alcian blue stain penetration was not a problem and en bloc staining could be used for discharged nematocysts. Following a 2 hr glutaraldehyde fixation, tentacles were washed in FSW and GDW and then either placed in 3% Alcian blue in 1 M sulfuric acid for 1 hr or pretreated in performic acid (prepared according to the methods of Pearse, 1968) for 10 min, then stained in Alcian blue for 1 hr. The tissue was washed, dehydrated in acetone, and embedded in Spurr embedding medium. Sections were poststained in uranyl acetate followed by lead citrate.

RESULTS

Capsule Formation

Cnidoblasts (nematocyst producing cells) are characterized by a large complement of rough endoplasmic reticulum (RER) (Fig. 1) and by a Golgi-microtubule complex in which a Golgi apparatus encircles a cage of approximately 100 microtubules (Fig. 2). The microtubules apparently originate from a single centriole (or the cytoplasm near the centriole) and can measure up to 7 μm in length (Fig. 3). The capsule primordium is surrounded by the microtubules and increases in length along the long axis of the microtubules in a direction leading away from the centriole.

Bristle-coated vesicles (75 nm in diameter) are visible in the cytoplasm between the trans face of the Golgi and the nematocyst (Fig. 3). The nematocyst is covered by a limiting membrane (Fig. 3). Lying directly beneath this membrane is a double wall consisting of an electron-dense, outer wall and an electron-lucent, inner wall (Fig. 3). Neither the outer wall nor the inner wall is visible in the end of the developing capsule nearest to the Golgi (Fig. 3). The outer and inner nematocyst walls are clearly visible in all other areas of the developing capsule (Fig. 3). The electron-dense, outer wall measures 17 nm thick (Fig. 3), and does not change in appearance or thickness throughout nematocyst development. In contrast, the electron-lucent, inner wall changes both in appearance and thickness throughout nematocyst development.

The early nematocyst capsule (e.g., that recently formed by the Golgi apparatus) has an inner wall that measures 60 nm thick (Fig. 3). The early capsule inner wall has a mottled appearance with occasional electron-dense areas within the predominantly electron-lucent wall matrix (Fig. 3). Small electron-lucent regions, tentatively identified as particles, are clearly visible in the electron-dense capsule interior (Fig. 3). These particles measure 8–20 nm in length and are similar in appearance to the capsule inner wall matrix (Fig. 3).

The capsule increases in length so that portions of the capsule extend beyond the cage of microtubules (Figs. 4, 5). Such portions of the capsule become wrinkled (i.e.,

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**Fig. 1.** Longitudinal section of a conventionally prepared cnidoblast filled with rough endoplasmic reticulum (rer). × 20 500.

**Fig. 2.** Cross section of an Alcian blue-prepared cnidoblast involved in capsule formation. The developing capsule (c) is surrounded by numerous microtubules (arrowheads) and a Golgi apparatus (g). × 42 500.

**Fig. 3.** Longitudinal section of a ruthenium red-prepared cnidoblast involved in capsule formation. Microtubules (mt, arrows) appear to originate from the centriole (ce) and surround the capsule primordium. Coated vesicles (cv) are visible in the cytoplasm between the Golgi (g) and the developing capsule. The capsule is covered by a limiting membrane (m). Note that the outer wall (cow) and inner wall (ciw) of the capsule are visible after the first micrometer of capsule length (arrow), beginning from the capsule-end nearest the Golgi. The inner wall has a mottled appearance. Electron-lucent particles (circles) are present in the capsule interior. × 56 500.
appear to undulate in thin section), whereas portions of the developing capsule enclosed by the microtubule array remain straight (Figs. 4, 5).

As capsule formation is completed, the Golgi-microtubule complex "moves off" of the capsule while simultaneously forming the external tube. At this point, the entire capsule appears to be wrinkled (Fig. 6) and the capsule inner wall has a mottled appearance (Fig. 7). However, at 70 nm, the inner wall measures 10 nm thicker than in the previous developmental stage.

As external tube formation proceeds, a large electron-lucent mass can be observed in contact with the capsule inner wall (Figs. 8, 9). The mass apparently has a particulate composition with individual 8- to 20-nm particles visible around its periphery (Fig. 8). The occurrence of the electron-lucent particulate mass is accompanied by capsule straightening, an increase in inner wall thickness from 70 to 77 nm, and by a change in the appearance of the inner wall. The inner wall now appears to be less mottled (i.e., more uniformly electron lucent) than in previous developmental stages (cf. Fig. 3 with Figs. 8, 9).

Following tube inversion into the capsule, the capsule inner wall dimensions increase further, and now measure up to 105 nm thick (Fig. 10). The capsule inner wall is uniformly electron lucent and no longer mottled (Fig. 10).

The capsule inner wall of mature undischarged and discharged nematocysts measures between 40 and 60 nm thick, depending on the individual nematocyst (Figs. 11, 12). Mature capsules have a uniformly electron-lucent inner wall.

**Tube Formation**

The external tube is formed by the Golgi-microtubule complex (Fig. 13) in a manner that is at least superficially similar to that of capsule formation. Like the early capsule, the early tube has a mottled inner wall. However, the early tube inner wall (at 13 nm) is only one-fourth as thick as that of the early capsule.

After the tube is formed, it inverts into the capsule. At this point, the external tube inner wall (equivalent to the inverted tube outer wall) is still mottled and measures 19 nm thick (Fig. 14), having increased by 6 nm from the previous developmental stage.
Once completely inverted into the capsule, the inverted tube outer wall is less mottled and measures 15 nm thick (Fig. 10), having decreased by 4 nm from the previous developmental stage.

The inverted tube outer wall of the mature nematocyst (also commonly referred to as a "thread" in the mature nematocyst) is not mottled and measures 11 nm thick (Figs. 11, 12), having decreased in thickness by 4 nm from the recently inverted tube.

**Mature Nematocyst Capsule and Everted Thread Inner Walls**

The nematocyst contents appear electron dense in Alcian blue preparations of undischarged nematocysts (e.g., Fig. 10). Hence, possible differences in Alcian blue staining of the wall components throughout nematocyst development could not be detected. Because the contents are expelled from holotrich nematocysts at discharge, Alcian blue staining properties of the mature capsule and thread inner walls are compared below using discharged nematocysts.

A conventionally prepared nematocyst (glutaraldehyde and osmium fixed, uranyl acetate and lead citrate poststained) is shown in Fig. 12. The thread-capsule junction is clearly indicated just below the opened apical flaps.

Following the incubation of discharged nematocysts in Alcian blue, electron-dense staining is apparent along the thread inner wall (Fig. 15). Staining is restricted to the thread and does not occur along the capsule inner wall (Fig. 15).

Pretreatment of fired nematocysts in performic acid prior to Alcian blue incubation results in electron-dense staining of the capsule inner wall in addition to the thread inner wall, which is stained as before (Fig. 16). Hence, the oxidation of disulfide bonds by performic acid results in increased staining of the capsule inner wall, but not of the thread inner wall. Performic acid-treated nematocysts have capsule inner walls that measure from 96 to 144 nm thick, and everted thread inner walls that measure 22 nm thick (Fig. 16). Recall that mature nematocysts that are not exposed to performic acid have capsule inner walls that measure from 40 to 60 nm thick, and thread inner walls that measure 11 nm thick. Hence, performic acid treatment apparently causes nematocyst inner walls to thicken.

**DISCUSSION**

The nematocyst is formed by a Golgi apparatus, which is positioned at one end of a microtubule cage. The developing nematocyst increases in length within the microtubule cage in a direction leading away from the Golgi. In the present study, we have shown that the nematocyst end nearest to the Golgi is membrane limited, but also appears to lack the inner and outer nematocyst walls. Hence, it is possible that membrane is added to the developing nematocyst before wall formation takes place. Typically, membrane is added to enlarging secretory products by vesicle fusion. Susi and co-workers (1971) described budding of small, coated vesicles from the Golgi and their subsequent fusion with the enlarging acrosomal vesicle in developing rat sper-
matozoa. In the present study, numerous coated vesicles were observed in the cytoplasm between the trans face of the Golgi and the developing nematocyst. The coated vesicles might originate from the Golgi and provide membrane by fusing with the developing nematocyst. The vesicle contents might provide other materials necessary for nematocyst formation.

Nematocyst Wall Formation

As was stated above, the nematocyst wall might form after the addition of membrane to the developing nematocyst. Perhaps wall precursors bind to the inner layer of this membrane to cause wall formation. Unfortunately, the electron density of the outer wall is similar to that of the nematocyst interior. Thus, possible outer wall precursors could not be identified in the present study.

Fortunately, the situation for inner wall formation is more clear cut because the predominately electron-lucent inner wall is starkly contrasted against the electron-dense nematocyst interior. The inner wall first appears approximately one micrometer from the end of the developing nematocyst nearest the Golgi, and is clearly visible in all areas of the nematocyst beyond this point. We propose that the end of the existing inner wall acts as an organizing center for fur-
ther wall formation. The electron-lucent particles observed in the nematocyst interior might be inner wall precursors that bind to the end of the existing inner wall to cause it to increase in length. Such an increase in length of the inner wall would occur in the opposite direction to the lengthening of the nematocyst (i.e., toward the Golgi rather than away from it). Because the newly formed inner wall has a constant thickness, it is unlikely that significant precursor binding to the wall surface occurs at this developmental stage.

**Nematocyst Inner Wall Growth**

Inner wall growth is defined as an increase in inner wall thickness accompanied by a change in the appearance of the inner wall from a mottled appearance to a uniformly electron-lucent appearance. The capsule inner wall is formed at 60 nm thick and increases in thickness throughout nematocyst development, possibly to a maximum of about 105 nm shortly after the tube inverts into the capsule. Likewise, the tube inner wall increases in thickness from 13 nm at tube formation to 19 nm thick at tube inversion. The increase in inner wall thickness in both the capsule and tube might result from an addition of material onto the existing inner wall surface. Perhaps the electron-lucent particles in the nematocyst interior provide precursors that bind to the inner wall surface to cause wall thickening. It is conceivable that the particles might have a greater binding affinity for the end of the existing inner wall than for the inner wall surface. Thus, at the completion of inner wall formation (i.e., the occupation of all wall-end binding sites), possible binding sites along the inner wall surface might then attract and bind a large number of particles, resulting in a thickening of the inner wall.

Another possible explanation for wall thickening is that the electron-lucent particles in the nematocyst interior might somehow become "sticky" at this stage of nematocyst development, and thus more readily bind to the inner wall surface than in earlier developmental stages. This idea might also help explain the occurrence of the large, electron-lucent particulate mass in contact with the capsule inner wall. The mass is apparently made up of thousands of electron-lucent particles that tend to clump together, as well as adhere to the inner wall surface. Schmidt (1981) described an electron-lucent material attached to the inner wall of developing octocoral nematocysts. The material diminishes as the octocoral nematocyst inner wall thickens.

Besides wall thickening, the inner wall changes from a mottled appearance to a uniform, electron-lucent appearance during wall growth. The mottled inner wall might be weakly crosslinked. Hence, the electron-dense material in the nematocyst interior might partially penetrate the inner wall, causing the wall to have a mottled appearance (i.e., the immature wall might be porous). With an increase in crosslinking, this electron-dense material might then be excluded, causing the inner wall to appear more uniformly electron lucent.

Another possibility is that the mottled wall might be incompletely polymerized. Wall maturation would include further polymerization and result in a uniform electron-lucent appearance of the wall.

**Nematocyst Inner Wall Maturation**

The inner wall of the mature nematocyst is approximately one-half as thick as that of the late-stage immature nematocyst (e.g., a nematocyst involved in tube inversion). Hence, inner wall maturation might involve a thinning of the inner wall. Following performic acid treatment, the mature nematocyst inner wall measures about as thick as that of the late-stage immature nematocyst. Thus, the oxidation of disulfide crosslinks apparently causes a wall thickening that restores the mature nematocyst inner wall to the dimensions of the late-stage immature nematocyst inner wall. It follows that a wall thinning during development might result from the formation of disulfide crosslinks among the inner wall molecules.
Recall that the change in the inner wall appearance from the mottled inner wall to the uniform, electron-lucent inner wall might be caused by disulfide bond formation. We speculate that the formation of disulfide crosslinks begins during inner wall growth (resulting in the change in its appearance) and continues during inner wall maturation to cause inner wall thinning.

A Possible Function for Microtubules in Nematocyst Development

The existence of a cage-like array of microtubules associated with the Golgi apparatus during nematocyst development has long been recognized (Slautterback, 1963; Westfall, 1966; Carré, 1972; Skaer, 1973; Holstein, 1981; Schmidt, 1981). However, the function of the microtubules in nematocyst development has remained obscure, perhaps because the Golgi is considered to be responsible for the formation of the capsule and external tube. Slautterback (1963) suggested that the microtubules might conduct water and other small molecules to the developing nematocyst. Westfall (1966) proposed that the microtubules might prevent random fusion of Golgi-budded vesicles with the developing nematocyst. Westfall also thought that the microtubules might provide support for the newly formed capsule wall until it matures (i.e., hardens).

Holstein (1981) observed that the number of microtubules surrounding the developing nematocyst changes with the diameter of the forming organelle. Holstein concluded that the microtubules might somehow regulate the diameter of the nematocyst.

We propose that the microtubules support the nematocyst during wall formation. With the aid of microtubule support, the particles presumed to be inner wall precursors might bind together to establish a properly shaped template of wall material. Recall that the nematocyst collapses (appears to be wrinkled) once it lengthens beyond the microtubule cage. However, following inner wall growth, the capsule straightens to regain its original shape. Perhaps the processes that occur during inner wall growth "strengthen" the template to aid in this straightening of the capsule. Thus, microtubule support might be critical during nematocyst wall formation so that a template of wall material can be formed with the proper shape.

Mature Nematocyst Capsule and Thread Walls

The thread inner wall of the mature nematocyst is stained by Alcian blue both prior to and after performic acid treatment. In contrast, the mature nematocyst capsule inner wall is stained only after performic acid treatment. Because Alcian blue stains "free" sulfur groups, it appears that the thread wall has free sulfur groups that are absent from the capsule inner wall. The capsule inner wall is stained by Alcian blue after performic acid treatment (which oxidizes disulfides to sulfonates). Thus, capsule inner wall sulfur groups are tied up in disulfide bonds that must be converted to free sulfur groups (e.g., sulfonates) before Alcian blue staining can occur. This observation has important implications with respect to the differing physical properties of the thread and capsule. Crosslinking of moderately flexible materials such as collagen causes the materials to become rigid. The collagen of the Achilles tendon of the mature rat is highly crosslinked (and inflexible), whereas that of the flexible rat tail is much less crosslinked (Stryer, 1981). While most vertebrate collagens are crosslinked by means of an aldol condensation between lysine residues (Tanzer, 1973), many invertebrate collagens including nematocyst wall collagen are crosslinked by disulfide bonds (Adams, 1978).

One possibility is that the incidence of free sulfur is inversely related to that of the disulfide bonds (i.e., the more free sulfur groups present, the fewer the disulfide bonds). Hence, the thread inner wall (with more free sulfur groups) would have fewer disulfide crosslinkages than the capsule inner wall. The greater flexibility of the thread...
than of the capsule (resulting in its ability to become highly folded within the capsule) might be explained by the possible occurrence of fewer disulfide crosslinkages in the thread inner wall. Perhaps the thread inner wall sulfur groups are in some way chemically modified so that they are inaccessible to the compound responsible for forming the disulfide bonds during development, and yet still able to bind with the Alcian blue.

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REFERENCES