

Active Nematocyst Isolation Via Nudibranchs

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Abstract Cnidarian venoms are potentially valuable tools for biomedical research and drug development. They are contained within nematocysts, the stinging organelles of cnidarians. Several methods exist for the isolation of nematocysts from cnidarian tissues; most are tedious and target nematocysts from specific tissues. We have discovered that the isolated active nematocyst complement (cnidome) of several sea anemone (Cnidaria: Anthozoa) species is readily accessible. These nematocysts are isolated, concentrated, and released to the aqueous environment as a by-product of aeolid nudibranch *Spirilla neapolitana* cultures. *S. neapolitana* feed on venomous sea anemones laden with stinging nematocysts. The ingested stinging organelles of several sea anemone species are effectively excreted in the nudibranch feces. We succeeded in purifying the active organelles and inducing their discharge. Thus, our current study presents the attractive possibility of using nudibranchs to produce nematocysts for the investigation of novel marine compounds.

Keywords Nematocyst · Sea anemone · Nudibranch · Venom · Natural products

Introduction

Cnidarians constitute the taxonomically most extensive group of venomous animals (Hessinger and Hessinger 1981). Every species possesses a unique complement of nematocysts, each potentially differing biochemically in venom composition as well as structurally (Endean and Rifkin 1975; Burnett et al. 1986). The nematocyst is a highly specialized, secretory, subcellular structure loaded with the venom it delivers (Lotan et al. 1995). It consists of a capsule containing a highly folded eversible tubule. The capsule is filled with a matrix of charged γ -glutamate polymers and cations generating high internal pressure (15 MPa) that drives discharge at accelerations reaching $5.41 \times 10^6 g$ ($g=9.81 \text{ m/s}^2$). The estimated pressure at the point of impact during discharge is more than 7 GPa, which is similar to the pressure exerted by bullets (Nuchter et al. 2006). The primary function of the penetrating type of nematocyst is the rapid delivery of complex mixtures of bioactive compounds, i.e., venoms, which can cause a variety of cytotoxic, neurotoxic, hemolytic, cardiotoxic, dermatonecrotic, immunogenic, and inflammatory effects (Yanagihara et al. 2002). Cnidarian venoms may include vasoactive compounds such as 5-hydroxytyptamine (5HT), catecholamines, histamine, and histamine liberators; neuroactive compounds such as quaternary ammonium compounds, certain amino acids and small peptides; and proteins including enzymes, such as proteases, phospholipases, and cytolytic or hemolytic agents (Chung et al. 2001). Due to these pharmacological properties, cnidarian venoms are potentially valuable tools for biomedical research and drug development (Anderluh and Macek 2002; Smith and Blumenthal 2007).

Cnidarian nematocysts are of added biotechnological interest, as an estimated 150 million people worldwide are

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exposed annually to jellyfish stings (Boulware 2006). Pure preparations of nematocysts may be useful in the development of repellents that prevent these stings (Lotan 2006). Furthermore, applications of cnidarian nematocysts as devices for delivering therapeutic, diagnostic, or cosmetic agents into a tissue are already being commercialized by at least one biotechnology company (Lotan and Eckhouse 2008; Lotan et al. 2008).

Accurate identification of the cnidome (the nematocyst complement of a species) is essential in the analysis of cnidarian venoms, as venoms may not only vary in composition between species but also between nematocyst types within a species (Burnett et al. 1986; Carrette et al. 2002). Unlike many other venom delivery mechanisms, such as those found in snakes and spiders, the quantity of venom flowing from any one nematocyst is incredibly small. As a result, in order to collect enough venom useful for analysis, many millions of nematocysts of the same type must be collected.

Aeolid nudibranchs have adapted over millions of years to live with and feed on nematocyst-armed cnidarians. During ingestion of their prey, the snails are able to inhibit discharge of nematocysts, possibly by the secretion of mucus (Conklin and Mariscal 1977) and via protective skin structures (Martin et al. 2007). Once ingested, a partitioning of undischarged nematocysts occurs. Some are incorporated and stored in the tips of the nudibranch cerata in organs termed cnidosacs by a process referred to as foreign organellar retention (Taylor 1983; Greenwood and Mariscal 1984). The cnidosacs with their nematocysts, the cleptocnidae, are thought to serve as a defense mechanism for the shell-less snails (Frick 2003). Greenwood and Garrity (1991) isolated nematocysts from nudibranchs, excising them from the cnidosac, a process that is tedious and not always successful. However, a large proportion of nematocysts is discarded intact and undigested in the nudibranch feces (Martin 2003). While masses of nematocysts are processed by aeolids, these organisms have not been previously considered as a source for obtaining the isolated and undischarged organelles. In this paper, we report the development of a method for isolation and partial separation of several active nematocyst types from the feces of the aeolid nudibranch *Spurilla neapolitana*.

Materials and Methods

S. neapolitana is a cosmopolitan species occurring in the Mediterranean, West Atlantic (from Florida to Brazil), and East Pacific (Baja California; Rudman 1999). It has been reported to feed on 37 sea anemone (Anthozoa: Actinaria) species (McDonald and Nybakken 1996). We cultured its distinct life cycle stages in a marine laboratory (Schlesinger

et al. 2009). The anemone preying stage survives for 120 days. During this period, it can be used to isolate stinging cells and will spawn a total of approximately 40×10^6 zygotes. Predation induces asexual reproduction of the

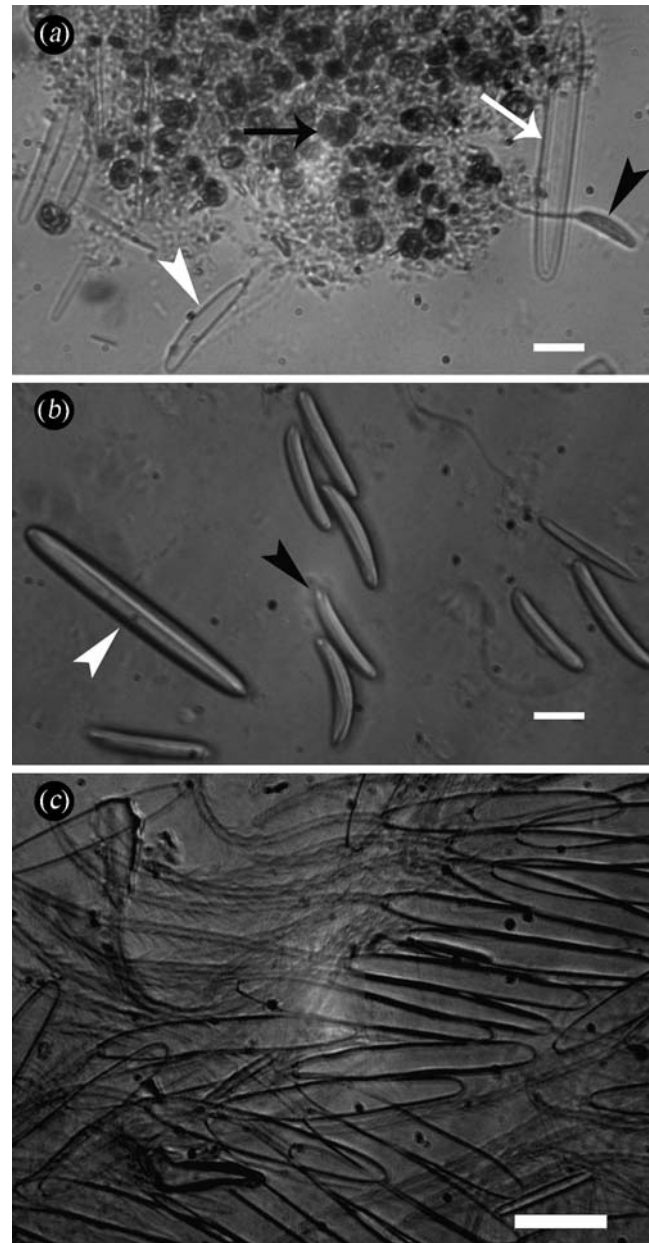


Fig. 1 Density gradient separation of nematocysts from *S. neapolitana* fecal pellets. **a** Discharged tentacular microbasic mastigophore (white arrowhead), acontial microbasic mastigophore (white arrow), spirocyst (black arrowhead), and algal (zooxanthellae) aggregates (black arrow) separated at S2 density interface (scale bar=10 μ m). **b** Acontial microbasic mastigophore (white arrowhead) and tentacular microbasic mastigophores (black arrowhead) separated below S3 density interface (scale bar=10 μ m). **c** Nematocyst discharge in 100 mM CaCl_2 , 0.22 μ m filtered seawater diluted 2:1 with distilled water. Note the empty capsules (upper right to center) and everted tubules (upper left). Scale bar=20 μ m

sea anemone *Aiptasia diaphana*, enabling self-sustained, continuous laboratory culture of *S. neapolitana* prey.

Prior to nematocyst harvesting, nudibranch cultures were starved for 24 h. The modular design of our culture system (Schlesinger et al. 2007) allows for the transfer of anemone-laden substrates into the nudibranch cultures. The anemones were rinsed in running seawater, whereupon they were transferred into a clean nudibranch culture apparatus. Nematocyst harvesting was executed from nudibranch pairs maintained in culture. Copulating pairs displayed higher predation rates and feces excretion. Fecal pellets were collected at 2 h intervals into 100 mM CaCl₂, 0.22 μm filtered seawater (CaFSW), vortexed, and kept at 4°C. The suspension was separated according to densities over a discontinuous Percoll (Sigma, USA) gradient, adapting a protocol developed for coral cell separation (Domart-Coulon et al. 2001). Percoll dilutions [30%, 50%, 70%, and 90% (vol/vol)] were prepared in CaFSW and layered in a 15-ml centrifuge tube. A 1-ml fecal suspension was loaded on top of the gradient, cooled on ice for 20 min, and centrifuged for 10 min at 280×g at 6°C. Nematocysts were collected with a fine pipette at the 0–30% (S0), 30–50% (S1), 50–70% (S2), and 70–90% (S3) density interfaces.

Results and Discussion

Feces of *Spurilla neapolitana* were negatively buoyant. When collected from the culture apparatus, they contained zooxanthellae, spirocysts, and four types of nematocysts: tentacular microbasic mastigophores (30 μm), acontial microbasic mastigophores (60 μm), acontial basitrichs (15 μm), and 5 μm microbasic mastigophores that appear to similar to mesenterial filament nematocysts. Most of the zooxanthellae separated between the S0 and S2 density interfaces. Spirocysts and discharged nematocyst separated at S2. Undischarged nematocysts of the types described above separated below the S3 interface (Fig. 1). To assess their viability and discharge properties, the isolated nematocysts were placed at a cell density of 600 nematocysts per microliter in a medium made up by diluting CaFSW medium 2:1 with distilled water. All nematocyst types isolated discharged on the cover glass (Fig. 1). Cultured in pairs in order to enhance predation rates, an average nudibranch culture will excrete approximately 1.5×10^5 active *A. diaphana* nematocysts a day, though this depends on nudibranch size, prey nematocyst composition, and water temperature.

In order to investigate the possibility of using *S. neapolitana* to isolate nematocysts from other anemone species, adult specimens were introduced to cultures of *Aiptasiogeton hyalinus*, *Anemonia viridis*, and *Actinia equina*. The distinct anemone species were readily preyed

upon by *S. neapolitana*, and similar quantities of their nematocysts (approximately 1.5×10^5 nematocysts per day) were found undischarged in the nudibranchs feces.

Nudibranchs feed on the polypoid benthic life cycle stages of all three Cnidarian classes: Hydrozoa, Scyphozoa, and Anthozoa (McDonald and Nybakken 1996; Ostman 1997). They are closely associated to their prey and use it for both food and shelter (Todd et al. 2001; Faucci et al. 2007). Therefore, collection and laboratory culture of nudibranch/cnidarian associations is rather straightforward. In the case of *S. neapolitana*, it provides direct access to the entire venom arsenal of at least four species. Thorough biochemical characterization of isolated nematocyst types using this gradient-based method could address the assertion that nematocysts differ in venom composition based upon their structural classification. We believe that using this type of approach for isolation of active nematocysts will advance the investigation of cnidarian venoms as a source of effective marine natural products.

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References

- Anderluh G, Macek P (2002) Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actinaria). *Toxicon* 40:111–124
- Boulware DR (2006) A randomized, controlled field trial for the prevention of jellyfish stings with a topical sting inhibitor. *J Travel Med* 13:166–171
- Burnett JW, Ordonez JV, Calton GJ (1986) Differential toxicity of *Physalia physalis* (Portuguese man-of-war) nematocysts separated by flow cytometry. *Toxicon* 24:514–518
- Carrette T, Alderslade P, Seymour J (2002) Nematocyst ratio and prey in two Australian cubomedusans, *Chironex fleckeri* and *Chiropsalmus* sp. *Toxicon* 40:1547
- Chung JJ, Ratnapala LA, Cooke IM, Yanagihara AA (2001) Partial purification and characterization of a hemolysin (CAH1) from Hawaiian box jellyfish (*Carybdea alata*) venom. *Toxicon* 39:981–990
- Conklin EJ, Mariscal RN (1977) Feeding behavior, ceras structure, and nematocyst storage in the aeolid nudibranch, *Spurilla neapolitana* (Mollusca). *Bull Mar Sci* 27:658–667
- Domart-Coulon IJ, Elbert DC, Scully EP, Calimlim PS, Ostrander GK (2001) Arogonite crystallization in primary cell cultures of multicellular isolates from a hard coral, *Pocillopora damicornis*. *PNAS* 98:11885–11890
- Endean R, Rifkin JF (1975) Isolation of different types of nematocysts from the cubomedusan *Chironex fleckeri*. *Toxicon* 13:375–376
- Fauci A, Toonen RJ, Hadfield MG (2007) Host shift and speciation in a coral-feeding nudibranch. *Proc R Soc B* 274:111–119

- Frick K (2003) Response in nematocyst uptake by the nudibranch *Flabellina verrucosa* to the presence of various predators in the southern Gulf of Maine. *Biol Bull* 205:367–376
- Greenwood PG, Garrity LK (1991) Discharge of nematocysts isolated from aeolid nudibranchs. *Hydrobiologia* 216:671–677
- Greenwood PG, Mariscal RN (1984) Immature nematocyst incorporation by the aeolid nudibranch *Spurilla Neapolitana*. *Marine Biology* 80:35–38
- Hessinger DA, Hessinger JA (1981) Methods for rearing sea anemones in the laboratory. In: Committee on Marine Invertebrates (ed) *Marine invertebrates*. National Academy Press, Washington, DC, pp 153–179
- Lotan A (2006) Compositions and methods for inhibiting nematocyst discharge. Nidaria Technology Ltd., Germany Patent Office
- Lotan T, Eckhouse S (2008) Methods, compositions and devices utilizing stinging cells/capsules for conditioning a tissue prior to delivery of an active agent. Nanocyte Inc., United States Patent Application Publication
- Lotan A, Fishman L, Loya Y, Zlotkin E (1995) Delivery of a nematocyst toxin. *Nature* 375:456
- Lotan T, Shaoul E, Eckhouse S (2008) Stinging cells expressing an exogenous polynucleotide encoding a therapeutic, diagnostic or a cosmetic agent and methods compositions and devices utilizing such stinging cell or capsules derived therefrom for delivering the therapeutic, diagnostic or cosmetic agent into a tissue. Nanocyte Inc., European Patent Office
- Martin R (2003) Management of nematocysts in the alimentary tract and in cnidosacs of the aeolid nudibranch gastropod *Cratena peregrina*. *Marine Biology* 143:533–541
- Martin R, Tomaschko K, Walther P (2007) Protective skin structures in shell-less marine gastropods. *Mar Biol* 150:807–817
- McDonald GR, Nybakken JW (1996) A list of the worldwide food habits of nudibranchs. <http://people.ucsc.edu/~mcduck/nudifood.htm>
- Nuchter T, Benoit M, Engel U, Ozbek S, Holstein TW (2006) Nanosecond-scale kinetics of nematocyst discharge. *Curr Biol* 16:R316–R318
- Ostman C (1997) Abundance, feeding behaviour and nematocysts of scyphopolyps (Cnidaria) and nematocysts in their predator, the nudibranch *Coryphella verrucosa* (Mollusca). *Hydrobiologia* 355:21–28
- Rudman WB (1999) *Spurilla neapolitana* (Delle Chiaje, 1823). Australian Museum, Sydney
- Schlesinger A, Goldshmid R, Hadfield MG, Kramarski-Winter E, Loya Y (2009) Laboratory culture of the aeolid nudibranch *Spurilla neapolitana* (Mollusca, Opisthobranchia): Life History Aspects. *Marine Biology* (in press)
- Schlesinger A, Kramarsky-Winter E, Loya Y (2007) Method and apparatus for propagating benthic marine invertebrates. Ramot at Tel Aviv University, United States Patent Application Publication
- Smith JJ, Blumenthal KM (2007) Site-3 sea anemone toxins: molecular probes of gating mechanisms in voltage-dependent sodium channels. *Toxicon* 49:159–170
- Taylor FJR (1983) Some eco-evolutionary aspects of intracellular symbiosis. In: Jeon KW (ed) *Intracellular symbiosis*. *Int. Rev. Cytol* 14:1–28
- Todd CD, Lambert WJ, Davies J (2001) Some perspectives on the biology and ecology of nudibranch molluscs: generalisations and variations on the theme that prove the rule. *Bollettino Malacologico* 37:105–120
- Yanagihara A, Kuroiwa J, Oliver L, Chung J, Kunkel DD (2002) Ultrastructure of a novel eurytele nematocyst of *Carybdea alata* Reynaud (Cubozoa, Cnidaria). *Cell Tissue Res* 308:307–318