First application of PDMPO to examine silicification in polycystine Radiolaria

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Abstract: 2-(4-pyridyl)-5-[4-(2-dimethylaminoethy lamincarbamoyl)methoxy]-phenyl oxazole (PDMPO) is a fluorescent compound that accumulates in acidic cell compartments. PDMPO is accumulated with silica under acidic conditions, and the newly developed silica skeletons show green fluorescent light. This study is the first to use PDMPO in polycystine radiolarians, which are unicellular planktonic protists. We tested Acanthodesmia sp., Rhizosphera trigonacantha, and Spirocyrtis scalaris for emission of green fluorescence. Entire skeletons of Acanthodesmia sp. and Sr. scalaris emitted green fluorescent light, whereas only the outermost shell and radial spines of Rz. trigonacantha showed fluorescence. Two additional species, Spongaster tetras tetras and Rhopalastrium elegans did not show fluorescence. Green fluorescence of the entire skeleton is more like the “skeletal thickening growth” defined by silica deposition throughout the surface of the existing skeleton. The brightness of the fluorescence varied with each cell. This difference in fluorescence may reflect the rate of growth in these cells. Green fluorescence in PDMPO-treated polycystines suggests the presence of similar metabolic systems with controlled pH. The results of this study shed light not only on the feasibility of using PDMPO in diatoms and siliceous sponges, but also in polycystines. Further application of PDMPO could refine polycystine skeletogenic hypotheses and offer new insight on the physiological functions of silica assimilation.

Key words: PDMPO, polycystine, radiolaria, silicification, skeletogenesis

Introduction

Polycystine radiolarians (unicellular planktonic Protista) have a very geometric, siliceous skeleton, with spherical, discoidal, spiral, and multi-segmented conical forms. As shown in Fig. 1A, some spherical polycystines have concentric, spherical shells (e.g., Order Spumellaria, Superfamily Actinommoidea), as if each of their shells grows from the inside, whereas multi-segmented conical polycystines (Fig. 1B) (e.g. Order Nassellaria, Suborder Cyrtellaria) appear as though they make their segments one-by-one (Hollande & Enjumet 1960, Petrushevskaya 1962).

The skeletogenesis of polycystines is little known. The form of skeletogenesis is generally hypothesized from observations of skeletal forming signs recorded in plankton and fossil specimens (e.g. Anderson & Swanberg 1981, Thurow & Anderson 1986, Nishimura 1990, Itaki & Björklund 2007). However, these studies examined the skeletogenesis only of fully grown polycystines, and the growing process from young to fully grown cells has not yet been observed. One of the most effective methods for studying polycystine skeletogenesis is to observe the growing process, but it is difficult because long-term culture experiments of polycystines have not yet been successful (e.g., Anderson 1983, Anderson et al. 1989, Matsuoka & Anderson 1992, Sugiyama & Anderson 1997).

One effective method for studying polycystine skeletogenesis is the 2-(4-pyridyl)-5-[4-(2-dimethylaminoethylaminocarbamoyl)methoxy]-phenyl oxazole (PDMPO; Molecular Probe, Lysosensor™ DND-160 Yellow/Blue) technique (Shimizu et al. 2001), which has been applied for investigations of the biological silicification of demosponges (Schröder et al. 2004) and diatoms (Hazellar et al. 2005). PDMPO is a fluorescent compound and an aci-
dotropic pH indicator that accumulates in acidic cell compartments. In diatoms, the silica deposition vesicle (SDV) is an acidic cell compartment (Vrieling et al. 1999). PDMPO accumulates with silica under acidic conditions in the SDV, and is then deposited in the skeleton. Due to PDMPO, the newly developed silica skeletons emit green fluorescence light.

Although the biochemical conditions and growth rate during silicification and skeletogenesis cannot be directly observed, the application of PDMPO-silica fluorescence is worth implementing. Because SDVs are likely to exist in the polycystines, based on transmission electron microscope (TEM) images of probable silica aggregate granules (0.2–0.3 μm in diameter) between the siliceous test and the inner membrane of the cytoplasm in Collosphaera globularis Haeckel (Family Collospheeridae) (Anderson 1981, 1994), we believe that PDMPO is applicable for staining the newly developed skeleton of polycystines. Our results clearly show the existence of an acidic cell compartment in radiolarian cells, and this compartment is likely related to silica deposition.

Materials and Methods

The material used in this study was collected from surface waters (ca. 4 m depth) on 25 November 2008 by a 3-min tow using the No. 3 plankton net of Matsuoka (2002) (a 2-m long net with a 44-μm mesh and a 50-cm square opening) at a locality (Station 081125; 26°35’41”N, 127°50’30”E) off Sesoko Island, Okinawa, southwestern Japan. This area is commonly affected by the warm Kuroshio Current. The temperature and salinity of the seawater were 24.6°C and 33, respectively. The collected samples were processed at the Sesoko Tropical Biosphere Research Center, University of the Ryukyus (STBRC). Terminology used to describe radiolarians follows Suzuki (1998) and De Wever et al. (2001).

The collected materials were stained with PDMPO and observed by the following method. As this is the first report to apply PDMPO to polycystine radiolarians, the detailed procedures are explained as follows (Fig. 2):

1) The collected material was filtered through a sieve with a 1-mm opening to remove >1 mm plankton, predominantly Copepoda, for keeping polycystines in good condition. Polycystines weaken and die in the presence of >1 mm plankton (Fig. 2A).

2) The filtered material was settled for a few hours in a bottle full of seawater, because polycystine radiolarians tend to mostly settle to the bottom.

3) A small portion of the settled radiolarians was removed from the bottles with a Pasteur pipette and transferred into a small glass tube with 16 mL of seawater filtered with a 0.45-μm membrane filter. (Fig. 2B).

4) The undiluted PDMPO (1 mM) was dissolved in 20 mL of percolated seawater. Dissolved PDMPO (2.5 μM, 4 mL) was added to the radiolarian tube (final concentration: 0.5 μM PDMPO) (Fig. 2D).

5) The tubes were incubated under an artificial day-night cycle (12 h day/12 h night) with red and blue LED lights for 24 h at 26°C (Fig. 2E).

6) The samples were fixed in the incubation tubes with 37.5% formalin solution (Wako Pure Chemical Industries) (final concentration: 2% formaldehyde).

7) The incubated samples were moved to petri dishes (Fig. 2F), and the polycystines were collected with a Pasteur pipette under an inverted light microscope (Fig. 2G). The collected cells were mounted on glass slides (Fig. 2H). To avoid crushing the radiolarians with a cover slip, white Vaseline was placed in a ridge to a height of 0.5 mm at the edge of the cover slip. After adding the Vaseline, the cover slip was attached to the slide (Fig. 2I).

8) The slide was observed with an epifluorescence microscope (AX-80, Olympus) with UV-excitation (WU: BP330–385, DM400, BA420), and images of selected cells were digitally captured with a CCD camera (DXM-1200, Nikon 150 ppi).

9) After observing the slide, the cover slip was removed with a box cutter, and the embedded cells were removed from the slide to the petri dish containing 50% chlorine bleach with a fine brush (Fig.
2J). They were immersed for 1h, to dissolve the soft bodies of the cells (Fig. 2K).

10) The cells were removed from the bleach with a fine brush to another petri dish filled with water for 1h, to remove the bleach (Fig. 2L).

11) The cells were removed from the petri dish with a fine brush, put on a tray to dry (Fig. 2M), and were observed using scanning electron microscopy.

**Results**

Experimental PDMPO treatment was applied to six polycystine cells from five species, *Rhizosphaera trigonacantha* Haeckel (Order Entactinaria, Family Rhizosphaerididae, based on De Wever et al. (2001)), *Acanthodesmia* sp. (Order Nassellaria, Family Acanthodesmiidae, based on De Wever et al. (2001)), *Spirocyrtis scalaris* Haeckel (Order Nassellaria, Family Artostrobiidae, based on De Wever et al. (2001)), *Spongaster tetras tetras* Ehrenberg (Order Spumellaria, Family Spongodiscidae, based on De Weber et al. (2001)), and *Rhopalastrum elegans* (Ehrenberg) (Order Spumellaria, Family Spongodiscidae, based on De Weber et al. (2001)). As a result, each of *Rz. trigonacantha*, *Acanthodesmia* sp. and *Sr. scalaris* cells emitted fluorescence. The other two cells of *Sg. t. tetras* and *Rp. elegans* did not emit green fluorescent light, although because of their stretched pseudopodia they were considered to be alive in the glass bottle.

**Rhizosphaera trigonacantha** Haeckel (Fig. 3A–D)

This cell has a large, spherical cortical shell (430 μm in diameter) with many short radial spines (Fig. 3A, B). The medullary shells (Fig. 1A) are hidden within the thick spherical intracapsulum (Fig. 3A, B). The cortical shell was brightly fluorescent at green wavelengths (Fig. 3C, D). The condition of the hidden skeletons within the intracapsulum was not recognizable.

**Acanthodesmia** sp. (Fig. 3E–I)

The skeleton consists of a large, D-shaped ring in the center of the test and a bilobate framework, but the illus-
trated cell faces the oblique distal end of the bilobate framework (Fig. 3E, F, I). Strong, green fluorescence is visible on the edge of the skeleton bars, while red fluorescence occurs in the symbiont algae (a in Fig. 3G, H), which appear as large, yellowish-orange spherules under normal light (Fig. 3G, H).

**Spirocyrtis scalaris** Haeckel (Fig. 3J–N)

This cell has a conical test with nine segments (Fig. 3J, K, L). This appearance is typical of most multi-segmented, conical Nassellaria since the early Triassic (ca. 245 million years ago). The conical intracapsulum is in the first to seventh segments and fluoresces bright bluegreen (Fig. 3M). The siliceous skeleton fluoresces green, but the light is blurred and much weaker than that in the intracapsulum (Fig. 3M).

**Discussion**

This study is the first to use PDMPO in polycystine radiolarians, and the results could shed light not only on the feasibility of PDMPO for polycystines, but also on its great potential in the study of silicification, because the tests of *Acanthodesmia* sp., *Rhizosphaera trigonacantha*, and *Spirocyrtis scalaris* emitted green fluorescent light to varying degrees (Fig. 3C, D, G, H, M).

The green fluorescent light emitted by polycystine shells,
similar to that seen in diatom shells (Shimizu et al. 2001), suggests that PDMPO was deposited on the polycystine skeleton. The fluorescent image of Rz. trigonacantha, without PDMPO, in Suzuki (2005) showed only blue auto-fluorescent light in the intracapsulum, and its test did not emit green fluorescent light. Other published fluorescent images of several polycystines without PDMPO treatment (Takahashi et al. 2003, Matsuoka 2007) showed blue, yellow, orange, and red auto-fluorescence of the soft body or symbiont, but no fluorescence from the skeleton.

The green fluorescence in PDMPO-treated polycystines suggests the presence of metabolic systems for silica polymerization with controlled pH, similar to that seen in diatoms (Shimizu et al. 2001). The PDMPO is accumulated with silica in acidic cell compartments of diatoms, and then deposited in the skeleton. The PDMPO accumulating on the polycystine skeletons suggests that the silica of the skeleton was deposited in a structure similar to the SDV. Our results suggest the presence of SDVs in polycystines, which was also suggested by Anderson (1981, 1994).

The brightness of the fluorescence varies with each species. The fluorescence of Rz. trigonacantha and Acanthodesmia sp. is bright (Fig. 3C, D, G, H), but Sr. scalaris was not as bright as other cells (Fig. 3M). The green fluorescent light of Rz. trigonacantha, Acanthodesmia sp., and Sr. scalaris suggest that these cells formed new skeletons after the addition of the PDMPO. The brightness of green fluorescent light reflects the concentration of PDMPO in the skeleton, and suggests differences in the accumulation rate of silica among cells. In contrast, Spongaster tetras tetras and Rhopalastrum elegans did not show any green fluorescent light. Both of them belong to the family Spongodiscidae. Matsuoka (1992) reported that Rhopalastrum truncatum Ehrenberg, which belongs to Spongodiscidae, developed its test step-by-step, and finally stopped growing. It is possible that the Sg. t. tetras and Rp. elegans we observed were not in a growth period or had stopped growing.

Further application of the PDMPO method could refine polycystine skeletogenic hypotheses and bring new insights into the physiological functions of silica assimilation. A previous study suggested that the concentric shell of the superfamily Actinommoidea develops by the addition of new spherical shells, while Cyrtellaria grows by making new segments (Hollande & Enjumet 1960, Petrushevskaya 1962, Itaki & Bjorklund 2007). This idea was verified by Suzuki (2006) who compared the various growth stages of the genus Sphaeropyle (Family Actinommaidae). However, we found that the entire skeleton showed green fluorescence, which means that polycystine shells grow by thickening their skeleton rather than by making new segments. The further application of the PDMPO method could resolve this problem.

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**References**


English abstract


