Journal of Experimental Marine Biology and Ecology 463 (2015) 8–16

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Cellular orchestrated biomineralization of crystalline composites on implant surfaces by the eastern oyster, *Crassostrea virginica* (Gmelin, 1791)

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ARTICLE INFO

Article history:
Received 3 September 2014
Received in revised form 1 October 2014
Accepted 11 October 2014
Available online 12 November 2014

Keywords:
Cellular biomineralization
Hemocytes
Exosomes
Oysters
Shell formation
Laser scanning microscopy

ABSTRACT

The phylum Mollusca is unmatched in the mastery of cellular-engineered shell microstructures, producing shells that are visually elegant, structurally complex and fracture resistant. Shell formation in the eastern oyster, *Crassostrea virginica* is a process that involves both hemocytes (blood cells) and the outer mantle epithelial cells (OME). This study reports the secretion of an extracellular matrix (ECM) and cellular activity during shell layer formation by observing folia and prismatic development on different metal alloy surfaces (Ti6Al4V titanium, 7075-T6 aluminum, and 316 L stainless steel) implanted under the OME. During prism formation, a close association was observed between the ECM surrounding the prisms, the OME, and hemocytes. The prismatic ECM walls appeared to originate from the OME surface and were auto-fluorescent. During folia formation, an initial infiltration response of hemocytes to the implant surface was observed followed by the formation of a thin translucent ECM membrane onto which exosome-like vesicles, some containing crystals, deposited. These structures progressively organized into well-defined folia mineral. It is proposed that molluscan shell is formed through a series of coordinated events involving cells and cell products whereby both organic and mineral phases are secreted, organized, and simultaneously formed. The coordination of cells and cell products for shell synthesis opens a new realm of cellular control not previously explored in shell formation.

1. Introduction

Shell formation in the eastern oyster, *Crassostrea virginica* is a cell-driven process that involves cells from two different tissues: 1) hemocytes (blood cells) of connective tissue origin which also function in immune processes (Humphries and Yoshino, 2003) and supply intracellular calcium carbonate crystals to the mineralization front (Fleury et al., 2008; Kadar et al., 2009; Mount et al., 2004); and 2) outer epithelial cells of the mantle organ (OME) which secrete the organic constituents of shell (Lowenstam and Weiner, 1989; Myers et al., 2007). These cells work cooperatively to produce a bio-composite that is composed of crystals embedded and imbued within an organic macromolecular complex, conventionally referred to as organic matrix (Lowenstam and Weiner, 1989). The organic matrix is comprised of proteins, peptides, lipids, and carbohydrates and is purported to control the organization of the mineral phase by extra-cellular means (Addadi et al., 2006; Lowenstam and Weiner, 1989). The present study describes the cellular orchestration of shell composites on metal alloy implants observed occurring directly at the mineralization front. Observations show that once an organizing membrane is in place, the organic and mineral phases form simultaneously and are organized by hemocytes and OME. Henceforth, the organic complex within which the mineral layers form is referred to as the extracellular matrix (ECM) to reflect the intimate involvement of these cells.

Oyster shell is a multi-layered composite primarily composed of calcite and about 1% total ECM (Rusenko et al., 1991). Two primary layers of shell microstructures are formed. A thin prismatic layer (~100 μm thickness) forms the outer shell layer and is comprised of adjacently positioned columnar, polycrystalline prisms enveloped in ECM. The prisms are oriented such that the long axis is perpendicular to the shell surface. An inner foliated layer forms 90% of shell mass and is comprised of ECM and flattened, regularly shaped crystal units or

Abbreviations: DIC, differential interference contrast; ECM, extracellular matrix; EDS, energy dispersive spectroscopy; FITC, fluorescein isothiocyanate; LSM, laser scanning microscopy; OME, outer mantle epithelium; PBS, phosphate buffered saline; RCF, relative centrifugal force; REF, refractive granulocytes; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TRITC, tetramethylrhodamine isothiocyanate.

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http://dx.doi.org/10.1016/j.jembe.2014.10.014
0022-0981/

laths, oriented with their long axes parallel but slightly inclined to the plane of the shell. These laths coalesce to form sheets or lamina (Carter, 1990).

In adult oysters, shell layer formation can be observed by notching the shell margin. In these studies, both prismatic and foliated layers are regenerated in the notched region and are initiated with the delivery of calcite crystals of intracellular origin to the mineralization front by refractive (REF) hemocytes (Mount et al., 2004). The first event necessary for layered shell formation is the provision of a substrate membrane. The oyster accomplishes this task by the secretion of an organic periostracal membrane from specialized epithelial cells located in the mantle organ of the mollusk (Checa, 2000; Myers et al., 2007). Most published accounts of shell formation have relied on *post hoc* observations of previously formed shell layers so little is known about the cellular aspects of the process. Without detailed observations at the mineralization front, the origin and role of cells and the ECM in shell layer formation have remained speculative and elusive. This report provides evidence that shell formation is indeed orchestrated by cells and that the ECM is multifunctional and likely an essential, signaling component in the shell formation process.

2. Materials and methods

2.1. Oyster collection and holding

Eastern oysters, *C. virginica* were obtained from Pemaquid Oyster Company Inc. (Waldoboro, ME, USA); oysters were 2–3 year old market select size, between 75–85 mm shell height. After receipt, the oysters were held in a 180 gal (681 L) tank at 18 °C in artificial seawater at 31 salinity with saturating levels of dissolved oxygen. The animals were fed twice a week with Shellfish Diet 1800® (Reed Mariculture Inc., Campbell, CA USA). Tank water was continuously filtered except for several hours during feeding. Experimental animals were kept in 50 gal holding tanks under the same conditions as the acclimation tank.

2.2. Preparation of metal alloy disks and foils for implantation

Polished (1 μm finish) titanium (Ti6Al4V-grade 5) and aluminum (AA7075-T6) and 316 L stainless steel disks (Ti and 316 L, Goodfellow Corp., Oakdale, PA, USA; Al, McMaster Carr, Aurora, OH, USA) measuring 12.5 mm in diameter by 1 mm thick were cleaned by a series of 5 min washes, first in acetone followed by isopropyl alcohol and ending with methanol. The disks were flash dried on a heat block prior to implantation into the oyster. Alternatively, metal alloy foils including titanium (Ti6Al4V), aluminum (AA7075-T6), and 316 L stainless steel were also tested. Each foil square measured 1 cm^2 by 1 mm thick (Goodfellow Corp., Oakdale, PA, USA). Foil inserts were cleaned and prepared in the same manner as the metal disks.

2.3. Implantation procedures

Implantation was accomplished by removing just enough of the posterior margin of the shell with a diamond saw so that the disks could be inserted into the extrapallial cavity (the region between the mantle tissue and the shell) just inside the most active margin of shell formation. In some cases, the disks were placed deep enough into the extrapallial cavity to come in contact with the adductor muscle region of the mantle. The adductor is the muscle responsible for maintaining valve closure. Implants remained in contact with the shell-facing side of the mantle organ throughout the duration of incubation time. Implants were collected at 3 h, 24 h, 8 day, 14 day and 28 day intervals. Square foils, when used, were placed into a "V" shaped notch which was cut into the shell margin using a tile saw with a diamond blade. The foils were glued in place using an ethyl cyanoacrylate based adhesive; glue prevented the ejection of the foils from the shell margin during normal mantle extension and retraction. Incubations extended up to 14 days and foil implants were excised from the shell with a scalpel prior to analysis.

2.4. Fixation, imaging and X-ray microprobe (EDS) analysis of implants

Immediately following removal, implants were viewed on a Nikon AZ-100 microscope using both fluorescein isothiocyanate (FITC) and epi-polarization channels at low magnification to verify the presence of a mineralized coating. After imaging, the samples were washed for 5 min in 0.2 μm filtered isotonic seawater. The samples were fixed for 1 h in 4% paraformaldehyde–0.1 M sodium cacodylate trihydrate buffer at pH 8.0. Following fixation, samples were washed 3× in 0.005 M sodium cacodylate trihydrate buffer pH 8.0, followed by dehydration through a graded series of ethanol washes starting with 25% ethanol in water, followed by 50, 75, 90 and 100% ethanol for 10 min each. After dehydration, the samples were critically point dried and sputter coated with platinum, and then visualized using a field emission 4800S Hitachi high resolution scanning electron microscope equipped with an Oxford INCA Energy 200 EDS (energy dispersive spectroscopy) and a GW Electronics Centaurus backscatter detector.

2.5. Fixation and imaging of oyster mantle sections

To obtain mantle tissue sections, live oysters were relaxed by injection of a 1% cocaine solution dissolved in molluscan phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM or higher NaCl, depending on the osmolality of the holding tank, pH 7.4) into the adductor muscle. Within 5 min the shells would gape, and the animal would be transferred to a cold solution of 10% paraformaldehyde in 0.1 M sodium cacodylate trihydrate buffer at pH 8.0. The animal was fixed overnight at 4 °C, the hinge ligament was manually opened and the flat valve was excised from the adductor muscle. Fixed relaxed mantle sections were dissected near the growing margin of shell. These sections were washed 3× in 0.005 M sodium cacodylate trihydrate buffer pH 8.0, followed by dehydration through a graded series of ethanol washes starting with 25% ethanol in water, followed by 50, 75, 90 and 100% ethanol for 10 min per wash. After dehydration, the tissue sections were critically point dried and sputter coated with platinum then visualized using a JEOL 5300 LV scanning electron microscope (SEM) or a Hitachi S4800 field emission SEM.

2.6. Imaging of live prismatic shell formation by epi-fluorescent and laser scanning microscopy

Notched adult oysters were held overnight in 50 gal holding tanks. Using a 3.8 cm 21-gauge sterile needle affixed to a 3 mL sterile plastic syringe, approximately 1 mL of hemolymph was removed from the adductor muscle of the intact animal and transferred to a 1.5 mL plastic microfuge tube. To fluorescently vital-label living hemocytes, 3 μM of a molluscan PBS buffered calcein AM ester solution (Invitrogen Life Technologies, Grand Island, NY, USA) was added to the tube and incubated for 1 h at room temperature. The tube bearing the cells was centrifuged at 3000 *g* (relative centrifugal force, RCF, at tip) for 3 min and the supernatant was discarded. The cell pellet was gently re-suspended and washed in molluscan PBS. The washed and labeled cell suspension was re-injected into the oyster adductor muscle and the animal was replaced into the aquarium. After 1.5 h incubation, the oyster was relaxed by injection of 1% cocaine solution (dissolved in molluscan PBS) into the adductor muscle. Within 5 min the shells would gape, and the hinge ligament was manually opened and the flat valve was excised from the adductor muscle. Live relaxed mantle sections were dissected near the growing margin of shell. To visualize live hemocytes on the growing prismatic layer margin, the shell facing side of the mantle was affixed to a glass cover slip and mounted to a glass slide. These slides were visualized on a Zeiss LSM-510 laser scanning microscope (LSM) using the Zeiss Plan Neofluar 40× oil 1.3 N.A. objective.