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Preserving the viscous coral surface mucus layer using low acid glycol methacrylate (GMA) resin

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Abstract

The surface mucus layer (SML) is of critical importance in health, disease, and stress responses of corals, however visualising the intact SML is challenging. Dehydration during histological preparation causes shrinkage and deformation of the mucus gel layer, while fragile, unattached mucus exudates are typically lost altogether. Here we describe a novel technique using water-soluble glycol methacrylate resin embedding that more accurately preserves the *in situ* SML. Thickness of the preserved SML is similar to that previously measured on live corals using a glass micro-probe. The technique allows microscopic visualisation of the SML structure, as well as thickness and continuity measurements, which are important indicators of SML function in health and disease.

Introduction

Mucus is produced by all phyla of the Animal Kingdom (Lang et al. 2016), and fulfils a number of functions including immunological, chemical and mechanical stresses, while allowing solute and gas exchanges across epithelial surfaces (Taherali et al. 2018). Brown and Bythell (2005) reviewed the various roles of mucus in corals, and it is clear that there are still many gaps in our understanding of the biology and ecology of coral mucus, including the dynamics of production and secretion, to the surface mucus layer (SML), as well as residence time within the SML. Understanding the structure and dynamics of the SML is fundamental to understanding the colonisation and maintenance of the coral microbiome in health and disease (Bythell and Wild 2011, Bakshani et al. 2018).

In humans and other vertebrates, thickness, biochemical composition, continuity, and physical structure of the SML have been found to be useful indicators of organismal health, and particularly in gastrointestinal and respiratory tract function (Atuma et al. 2001; Bansil and Turner 2018; Taherali et al. 2018). However, there are a number of challenges to accurately preserving and quantifying the in vivo SML thickness and physical state in aquatic invertebrates such as cnidarians. Firstly, enhanced mucus secretion is a rapid stress response (Brown and Bythell 2005), so fixation of samples must be extremely rapid. Secondly, at least part of the external SML is unattached, low-viscosity gel and highly fragile, so excessive water motion and physical handling must be avoided. Thirdly, dehydration, as used in routine histological processing, will likely cause shrinkage and deformation of the SML so samples must be maintained in their original hydrated state.

I*n-situ* measurements using a glass microprobe by Jatkar et al. (2010) suggest that the thickness of the coral SML can be as much as 700µm in some species. Previous estimates of coral mucus thickness using volumetric calculations of the total mucus released (Wild et al. 2005; Koren and Rosenberg 2006), suggest a thickness of 0.3-3.8mm/ hr for *Acropora* sp. While several histological studies have succeeded in visualising a SML in corals (e.g. Marshall and Wright 1993, Marshall and Clode 2004), it is unknown how accurate these preservation techniques have been.

Here we describe the application of low-acid glycol methacrylate (GMA) resin to preserve the coral SML. GMA embedding was used as it is a water-miscible resin, which enables sampling and preservation without exposure to air and allows samples

to be maintained continuously hydrated throughout the embedding procedure (Cole 1984). We used rapid microwave fixation to reduce as far as possible the release of surface mucus as a stress response during the early fixation stage, and a system for sample collection that preserves the fragile SML by avoiding manual handling or excessive water motion around the sample. SML thickness and other indicators, compared to *in vivo* measures and observations (e.g. Jatkar et al. 2010), suggest that this technique accurately preserves the *in situ* SML structure.

Materials and Methods

A double-chambered polypropylene container was designed with the two chambers separated by plankton mesh and screwcaps to each chamber to allow sample collection and processing without disrupting the SML (Fig. 1 and Electronic Supplementary Material). Coral samples were collected from Ko Phuket, Thailand and Heron Island, Australia. Corals cores (~2.5cm diameter) were extracted from massive colonies *Coelastrea aspera, Galaxea fascicularis, Porites lutea, Favites abdita*, and fragments (< 4.5 cm length) from *Acropora aspera* and *A. muricata*. These were attached to a screwcap using underwater epoxy (Fig. 1), and left submerged for 3-5 days in the field to acclimatise and allow for lesion healing. The sample container was uncapped at both ends and air bubbles removed before gently lowering over the coral sample and fastened to the screwcap securing the coral into the chamber (Fig. 1). The container was then capped at the other end and the fully enclosed coral carefully transported to the laboratory within 5 min.

Fixation, Embedding and Section Preparation

The sample was rapidly fixed inside the collection container using a conventional 1800W GE microwave oven set at 30% power following an adaptation of the method in Login and Dvorak (1993). This fixation technique effects tissue shrinkage less than formalin fixation (Leong et al. 1985). A 300 ml beaker of tap water was first microwaved for at least 2 min to heat the magnetron and power supply (Login and Dvorak 1993). After pre-warming, the beaker was removed, and replaced with the coral sample chamber in an ice bath. The ice bath provided the same function as the beaker with water, but also ensured that the chamber with the coral sample remained <10°C during this primary fixation process. Microwave fixation was conducted using 30 sec bursts until the coral was unresponsive to touch (max 4 min).

A 25mm diameter Whatman nucleopore filter disc (pore 0.2 µm, Fisher Scientific 09-300-62) and stir bar (4.5 mm) were added to the remote chamber of the sample container to improve mixing of reagents between chambers, but without allowing visible currents to occur in the chamber housing the coral sample. Secondary fixation was conducted by adding room temperature 8% paraformaldehyde in 0.2 µm filtered natural seawater (PF/FSW) into the remote chamber, resulting in ~4% PF/FSW in the whole container. The solution was gently stirred by placing on a Nuova stir plate (~60 rpm) for 10 min then left unstirred overnight at 4°C. The fluid in the remote chamber was then replaced with FSW three times using a syringe with tubing, each time gently mixing on the stir plate as before.

The coral was then embedded in a fume hood with low acid GMA resin (#02640-AB kit for light microscopy SPI Supplies, USA). Using a syringe with tubing, the FSW in

the remote chamber was replaced once a day for four days with 100% unpolymerized GMA/polyethelene glycol 20:1 v/v and 1.429 mg/ml benzyol peroxide catalyst at room-temperature. After each change, the remote chamber was stirred (~60 rpm) for 30 min, and the container placed in a L/S PTFE diaphragm vacuum to remove all visible air bubbles before leaving overnight at 4°C to infiltrate the tissues. After 4 repeat treatments, the GMA resin was polymerised at 40°C (Binder FED 400 oven) for 24 h followed by 24 h at 60°C. Once cooled to room temperature, the embedded coral was cut from the container using a handheld hacksaw (TPI 24).

It was not possible to decalcify the samples without deformation of the resin, so ~5 mm thick sections were prepared by cutting rough sections with the hacksaw and polishing a face with a series of wet/dry sandpaper (220, 400, 800, 1000, 1500, and 2500 grit). The polished surface of the section was then fixed to a glass microscope slide using cyanoacrylate glue and the exposed surface was then polished as above to produce ~0.5 mm thick sections.

PAS-AB and DAPI Staining

Mucopolysaccharides were identified using periodic acid-Schiff/Alcian blue (PAS-AB) staining, adapted from Strugula et al. (2008). Each section was briefly placed face down in deionized water, followed by a 2-min incubation in 3% acetic acid, then stained in Alcian blue solution (1% Alcian blue 8GX in 3% acetic acid, pH 2.5) for 2 h. After rinsing with 3% acetic acid then deionized water, the blocks were incubated in 1% aqueous Periodic Acid for 5 min, followed by another rinse in distilled water. The blocks were then placed in Schiff's reagent (Sigma 3952016) for up to 15 min, after which the excess reagent was removed with distilled water and the sections left to dry.

Sections were then mounted with glass coverslips using histoclear. Images of the mounted sections were captured using an Olympus E300 8MP digital SLR camera, and colour-corrected in Photoshop CS6.

Some sections were stained with DAPI (4′,6-diamidino-2-phenylindole) in filtered phosphate buffered saline (PBS, pH 7.4, 5 µg/ml) for 15 min in the dark, after which they were rinsed with filtered PBS. Antifade oil (CitiFluor AF1) was placed on the surface, followed by a coverslip and sealed with clear nail polish. The section was then observed using a confocal microscope (Nikon A1R confocal), with images processed and post-processed using the Nikon Elements and Photoshop software respectively.

Mucus thickness measurements

Measurements of SML thickness were made on PAS-AB stained sections using Image J software. Total SML thickness was measured perpendicular to the coral epidermis every 50 µm between corallites until 30 measurements (to 3 decimal places) were recorded. 3-5 embedded corals per species were measured and the average thickness (±SE) per species was calculated. Discontinuity (% pts with 0 measures/total # measures) were calculated (adapted from Strugala et al. 2008).

Results and Discussion

Preservation of mucus

Some of the GMA-embedded corals were observed with strings and/or streams of mucus, that appeared to be very similar to *in situ* viscous mucus strings observed from tidally stressed *Acropora* corals in the field (Fig. 2). PAS-AB staining of those sections showed significant concentrations of mucopolysaccharide in the exuded strings and in

adherent material (Fig. 2), thus confirming the ability of the embedding process to preserve both adherent and detached elements of the intact SML. Critically, comparable SML thickness (±SE) was measured in the preserved SML in the present study (390.2±12.9 to 634.8±22.2 µm) to that reported for live corals by Jatkar et al. (2010) using a glass microprobe technique, where values ranged from 145 to 700 µm for several identical species at the same study locations (Fig. 3).

Structure of the SML

The preserved SML was highly variable in presentation and appeared to consist of both adherent and outer, unattached or loosely-attached mucus layers. In fact, the coral SML bears a strong resemblance to the human upper gastrointestinal tract where there is an adherent mucus gel layer, over which sits a second layer (Atuma et al. 2001; Bansil and Turner 2018; Taherali et al. 2018). At least one of these layers may be produced by mucus found streaming from through the oral cavity (Fig. 4), though further studies are required to ascertain the source of the mucus. The inner and outer coral SML thickness are also comparable to the human GI tract mucus layers where the more proximal layer to the epithelium measures up to 150 µm, and the outer layer extending up to 700 µm (Bansil and Turner 2018).

Different layers within the coral SML showed variable PAS-AB staining due to the presence of acidic (containing carboxyl and/or sulphonic acid groups in the oligosaccharide side chains), staining blue, or neutral mucins, staining magenta (Ali et al. 2012). These observations suggest that different mucin types are produced within different layers in the coral SML, although a consistent staining pattern was difficult to determine. For *C. aspera*, a clear acidic adherent mucus layer was present, with a

potentially mixed upper layer (Fig. 5). For other species such as *Porites* and *Galaxea* that are known to produce mucus sheets, successive neutral mucin sheets were visible (Fig. 5). Also similar to the human distal gastrointestinal tract (Taherali et al. 2018), the adherent inner layer in corals such as *C. aspera,* appears to be bacteria-free as evidenced by DAPI staining (Fig. 5).

The continuity of the mucus layer has been used to determine human health such as in the GI tract in Crohn's disease (Strugala et al. 2008). With the more accurate preservation of coral SML afforded by the present study, it is possible to assess the continuity of the mucus layer. The normal human colon has a 1±0.7 % discontinuity, where ulcerative colitis (UC) conditions ranged from quiescent (1.6±0.9%) to severe active UC (25.7±8.8%) (Strugala et al. 2001). In this study, discontinuity in healthy corals (GMA embedded) ranged from 0% (*A. muricata*) to 14.4% (*C. aspera*). While the ecological significance of these measurements is unknown, discontinuity of the SML may allow exposure of the underlying epithelia to potential pathogens and further studies are needed to elucidate the coral health-disease implications of these observations.

In conclusion, the application of low acid GMA resin for embedding coral specimens represents an accurate technique to determine *in situ* mucous thickness by preserving the structure of the intact SML. This is in part due to use of a water soluble resin, rapid fixation, and avoidance of excessive motion around the corals.

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Figure Legends

Fig. 1. Design of double-chambered polypropylene pot for embedding of coral (a), where the coral is attached to the cap (b). Upon collection, the pot is screwed directly on to the cap (c) ensuring that no air bubbles are trapped.

Fig. 2. Coral mucus (arrows) streaming from *Acropora* spp noted in the field (Phuket, Thailand) and confirmed using GMA resin (a) *in situ* mucus strings from *Acropora sp.*, (b) – (e) showing GMA preserved corals with streams. (e) PAS-AB staining of GMA blocks confirming coral streams in GMA blocks are mucus. Scale bars = 1cm.

Fig. 3. Comparison of mean mucus thickness (±SE) between live (black) and GMA-embedded (white) healthy corals from Phuket, Thailand. No statistical differences observed (2-factor ANOVA, p=0.109).

Fig. 4. Discrete layers of mucus in *Coelastrea aspera* (a) embedded using GMA resin, and (b) typical histological preparation (permission from Brown and Bythell (2006)). Mucus (IM) is seen projecting through the coral mouth (CM), with a clear outer mucus layer (OM). Scale bar for (a) and (b) are 1 mm and 50 µm respectively.

Fig. 5. GMA preserved (a) *Coelastrea aspera* and (b) *Galaxea fascicularis* capturing existence of inner mucus (im) and outer (om) layers, and inner sheets (is) and outer sheets (os) of mucus. Extent of breaks in the mucus layer (dm) can be used to calculate the discontinuity %. A bacteria-free adherent layer is also evident by the void seen with the use of DAPI staining (c) with extent of layer shown by white arrow. Scale bars for (a) and (b) = 1 mm, and (c) = 300 µm.