LEARNING TO GLUE UNDERWATER: INSPIRATION FROM THE DECORATOR WORM

by

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Abstract

*Diopatra cuprea* is a predatory polychaete worm that constructs a mucus tube in the sediment and decorates a portion of the tube above the sediment with primarily shells, fiber, and algae. The decorated portion is a micro-reef that attracts prey for the sedentary predator. I characterized the tube and biological glues that the worm uses. The tube is composed of a proteinaceous bioadhesive rich in phosphates. We hypothesize that phosphates are adhesive promoters. I tested the worm’s viability as a new model system for studying biological adhesives and tested for protein/phosphate presence in bioadhesive. Proteins and phosphates were obvious in adhesive attached to clean glass beads. Adhesive lacked glycoprotein. The decorator worm has potential as a model for studying biological adhesives.

Introduction

Biological adhesives are ubiquitous in aqueous environments. Conserved molecular mechanisms in underwater adhesion can provide evolutionary insights into underwater adhesion across taxa. Natural underwater adhesive biochemistry provides insights into how to bioengineer an industrial glue to fix leaks, secure underwater sensors, or repair wet living tissue in medical and applied sciences (Stewart *et al.*, 2011). Others study underwater adhesion to prevent macrofouling on ship hulls (Rittschof, 2011; Tribou & Swin, 2009) or bacteria on implants (Reddy *et al.*, 2011). Marine invertebrates are common underwater adhesive producers. Mussel, sandcastle worm, caddisfly, sea cucumber, midge larva, and barnacles are all animals that use proteinaceous bioadhesive (Stewart, 2011, Kamino, 2010).
The bioadhesive produced by the common intertidal polychaete the Decorator worm (Diopatra cuprea) is described as mucus in scientific literature without published biochemical evidence. Decorator wormss build a microreef on the outside of their tubes that functions as a food-catching tool (Mangum et al., 1968; Woodin 1978). If the microreef is removed, worms rapidly rebuild tubes with peripheral decorations, including glass beads and other materials, such as whip coral, mixed ion exchange resin, synthetic ion exchange resin, strongly basic anion exchange resin, aminopropyl silane modified glass particles (.5-10 μm), imitation seagrass, plastic zip ties, iPhone cases, silicone, silicone infused with octamethylcyclotetrasiloxane (D4) and silicone infused with decamethylcyclopentasiloxane (D5). *D. cuprea* cured adhesive is easily accessible on glass beads and antifouling materials. We hypothesized that, as is the case for other polychaete worms, decorator worm bioadhesive is highly proteinaceous and phosphorylated with low amounts of glycoprotein. We hypothesize that decorator worms adhesive strength will decrease when decorating with noxious common antifouling agents of silicone and silicone loaded with the cyclic siloxanes D4 and D5.

**Materials**

**Worm Collection**

*Diopatra cuprea* were dug from the sediment by quickly inserting a shovel vertically into the sediment 5-10 cm from tube and pulling up the sediment and tube. The tube was extracted from the sediment by hand, rinsed, and placed in a bucket. In the laboratory, cleaned worm tubes were observed in water for extended sensory cirri indicating an occupied burrow. Occupied tubes were isolated in individual containers. Worms were collected within a km of 34°43’5” N 76°40’16” W.
Worms were maintained in glass jars 13 cm in radius with 900 mL of seawater at a constant temperature of 21°C and salinity of 31.5.

Plastic petri dishes 50 mm in diameter were made into collars by melting a 1 cm diameter hole in the center of dish with a soldering iron. The collars were used to isolate the worms from the sediment and enable placement, restriction, and specification of tube building materials.

**Reagents and Materials**

Phosphate Colorimetric Assay was purchased from Sigma-Aldrich.

Octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) were purchased from Sigma-Aldrich. Non-reinforced silicone sheeting (Code SH-2001-040) 0.040” Gloss was purchased from BioPlexus.

**Methods**

**Collection of D. cuprea glue**

Silica beads were used to collect glue samples. Silica beads were combusted at 500°C for 4 hours, cooled, and exhaustively rinsed with deionized water. In order to collect glue, worms were given isolated materials, such as silica beads, for decoration. Ten worms in tubes were fitted with plastic petri dish collars (Plate 1) with silica beads inside the petri dish. After tube tops were clipped, worms decorated with silica beads within 24 hours. Beads glued onto the tube and beads not glued into the tube (background controls) were collected.

**Plate 1. Worm fitted with plastic petri dish collar**
Glass beads were placed in the petri dish.

If the worm tube was too short to fit inside the petri, the silica beads were placed in the jar next to the worm. About 60 silica beads were placed in each petri dish or jar. After 24 hours, the newly built tube tops were clipped with scissors and collected with forceps. Background beads or beads that the worm did not decorate with were collected. The undecorated portion of the tube was clipped from 10 tubes with scissors.

**Observation of Glue on Beads**

Newly built tube tops and background beads were rinsed with deionized water 3 times in excess and inspected under a dissecting microscope. Individual beads with large clumps of glue were selected and examined under dissecting microscope. The tube top containing the beads was disaggregated. Glued and background beads were rinsed 2 more times with deionized water. Individual beads were used in tests.

**Coomassie Blue Staining**

Ten each of glued, background beads, and undecorated tube were photographed under microscope camera and stained in filtered 0.025% Coomassie Blue R-250, 7.5% Acetic
Acid, and 50% Methanol in NanoPure water for 1 hour. Samples were destained in 7.5% acetic acid overnight and photographed again.

**Alcian Blue Staining**

Glued and background beads were photographed under microscope camera and stained in 0.5% Alcian Blue solution in 3% Acetic Acid (pH 2.5) for 4 hours. Samples were destained in 3% acetic acid overnight and photographed again.

**Phosphoproteins**

The Phosphate Colorimetric Assay Kit (Sigma-Aldrich) was used to test for phosphate presence in the glue on glass beads, background beads, and undecorated tube. A 0.1 mM Phosphate Standard/ 200 µL ultrapure water was used to create a serial dilution from 0 to 5 nmole/well standards, increasing in 5% increments, in well rows of a 96 well plate. Glue-spotted glass beads, background beads, and undecorated tube were added to each well. 30 µL of Phosphate Reagent was added to each well, mixed well with a pipette, and incubated for 30 minutes at room temperature covered from light. Samples were removed from each well. Absorbance was measured at 650 nm using Spectramax® m2 Multi-detection Readers (Molecular Devices) spectrophotometric multiwell plate reader. Glue was examined under a dissecting microscope to see if phosphate entered the glue or if it was on the surface.

The amount of glue on beads and tube samples was quantified. Glass beads, background beads, and tubes were rinsed with deionized water, dried at 70° C for 2 hours, and weighed to the nearest 0.0001 g. To remove organic bioadhesive, glass beads and tubes were combusted at 500° Celsius for 4 hours. After combustion, the remains were cooled to room temperature, and reweighed to the nearest 0.0001 g.
**Testing decoration limits**

We qualitatively explored which substrates the worms would adhere to its tubes. Eleven worms in tubes with the top decoration removed were fitted with plastic petri dish collars 5 cm in radius with a 1 cm hole in the middle. Decorations added to each petri dish include: whip coral, mixed ion exchange resin, synthetic ion exchange resin, strongly basic anion exchange resin, aminopropyl silane modified glass particles (.5-10 µm), imitation seagrass, plastic zip ties, iPhone cases, silicone, silicone infused with octamethylcyclotetrasiloxane (D4) and silicone infused with decamethylcyclopentasiloxane (D5). After 24 hours, newly built tube tops were clipped with scissors and collected with forceps and photographed.

**Antifouling Substrate**

Substrates for antifouling tests were 90 circular ~6 mm diameter pieces hole-punched from non-reinforced silicone sheeting (Code SH-2001-040, BioPlexus) 0.040” Gloss and weighed to the nearest 0.0001 g. Two photographs were taken of each of the pieces to measure diameter (Plate 1) and height (Plate 2) of the pieces using ImageJ. The left, center, and right height was measured and averaged. Thirty pieces each were soaked in the cyclic siloxanes octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) for 24 hours then blotted and weighed to the nearest 0.0001 g. Photographs of each of the pieces infused silicone and control pieces were taken from the same angles as previously to measure the diameter and height.

**Plate 2. Diameter photograph example**
This photograph shows the diameters (red line) of 3 pieces.

**Plate 3. Height photograph example**

Left, center, and right heights are in red.

The volume of each of piece prior and post-infusion was calculated by using \( \text{Volume} = \pi \times (\text{radius}^2) \times \text{(height)} \). All of the pieces besides for the control group, were rinsed with
methanol, deionized water, and blotted dry with a paper towel to prevent the worm from gaining excess exposure to D4 and D5, especially prior to decoration.

**Obtaining Worms**

Worms were delivered overnight from Duke University’s Marine Lab (Beaufort, North Carolina) to Allegheny College (Meadville, Pennsylvania). Worms were maintained in glass jars 13 cm in radius with 900 mL of seawater made from Instant Ocean at a constant temperature of 21°C and salinity of about 31.5.

**Antifouling Substrate Decoration**

Adhesion strength when decorating with shell, silicone, silicone infused with D4, and silicone infused with D5 was tested by having 3 worms decorate in each category (n=12 total). 12 worms in tubes with the top decoration removed were fitted with plastic petri dish collars 5 cm in radius with a 1 cm hole in the middle. 10 of each decoration were placed in the petri dish. After 24 hours, the newly built tube tops were clipped with scissors and collected with forceps. The number of pieces of decoration remaining was counted.

**Observation of adhesive on silicones**

Newly built tube top decorations were examined and photographed with a microscope to measure adhesive surface area and qualitatively describe adhesive.

**Adhesive Strength**

The adhesive strength was calculated based on the following equation:

\[ \text{Adhesion Strength} = \frac{\text{Force}}{\text{Basal area}} \]

Force (N) was calculated by multiplying the weight of the dry decorations by the acceleration of gravity since the decorations immediately fell off the tube upon picking
up the tube cap with tweezers. Adhesive force between silicone glued to silicone was measured by pulling the pieces apart with a pulley system (Plate 17). A piece of thread connected the silicone decorations to a pulley in which weight was added to determine adhesive force. The thread was attached to silicone with Gorilla Glue on an area free of worm glue. The silicone decoration was held down using tweezers. Adhesive basal area (mm$^2$) was measured in the microscope photographs in ImageJ. Adhesive surface area was measured and qualitatively described from microscope photographs.

Results

Observation of Glue on Beads

Newly built bead tube tops were examined under a microscope for large clumps of glue.

Plate 1. Beads with Glue Patch

As compared to unglued beads (A), the glue is brown/copper in color and is on many sides of the bead (B).

Alcian Blue Staining
Alcian blue staining indicating presence of glycoproteins was found in small quantities on the glued beads (Plate 2, B) and in the undecorated tube (Plate 6) as compared to the background beads (Plate 2, A)

**Plate 2. Alcian Blue staining**

Red arrows point to small quantities of glycoproteins on the glued beads (B). Glycoproteins are not present on the outside of the background beads (A).

**Plate 3. Alcian Blue staining on tube**

Red arrows point to small quantities of glycoproteins on the tubes (Plate 3, A). Glycoprotein was not present on the inside of undecorated tube (Plate 3, B).
**Coomassie Blue Staining**

Beads with large clumps of glue were stained with 0.00% Brilliant Blue-G250 (Fisher BioReagants) and destained overnight.

**Plate 4. Beads Stained with Coomassie Blue**

Background beads do not have dense blue protein mass (Plate 4, A). The glue stained blue shows protein presence (Plate 4, B).

**Plate 5. Undecorated portion of tubes**

Most of the undecorated tube was covered in sand as showed in the unstained tube (Plate 5, A). The stained tubes showed great protein presence (Plate 5, B).
Plate 6. Stained glue on sand

Spots of glue stained with Coomassie can be seen on the outside of sand (Plate 6)

Plate 7. Undecorated tube stained with Coomassie

An undecorated portion of the tube not heavily covered in silica stains dark blue (Plate 7, A). Tube inside stains dark blue (Plate 7, B).

Phosphates

The concentration of phosphate (C) equals the amount of Phosphate in bioadhesive (nM) from standard curve (Sa) divided by the sample volume (Sv) in μL added to the well.

\[ C = \frac{S_a}{S_v} \]
Bars are the mean of the concentration of phosphate in nmol/well in background beads, glued beads, and the undecorated portion of the tube. Error bars are standard error of the mean. (n=30)

**Testing Decoration Limits**

After 24 hours, worms decorated with all of the following substrates: mixed ion exchange resin, synthetic ion exchange resin, strongly basic anion exchange resin, aminopropyl silane modified glass particles (.5-10 µm), imitation seagrass, plastic zip ties, iPhone cases, silicone, silicone infused with octamethylcyclotetrasiloxane (D4) and silicone infused with decamethylcyclopentasiloxane (D5). The worm did not decorate with whip coral. Whip coral has been observed on *D. cuprea* tubes in the wild (personal observation, 2016, Plate 8). The worm that decorated with Mixed ion exchange resin exited tube at about 23 hours and adhered itself to the petri dish.

**Plate 8. Whip coral and plastic in natural environment**
The red arrows are pointing to a blue piece of plastic (left) and an orange Whip Coral fragment (right).

**Plate 9. Mixed ion exchange resin under microscope**

**Plate 10. Synthetic ion exchange resin under microscope**
Synthetic ion exchange resin tube caps.

Plate 11. Strongly basic anion exchange resin

Plate 12. Aminopropyl silane modified glass particles (.5-10 µm)
Plate 13. Imitation seagrass

Plate 14. Plastic zip ties

Plate 15. iPhone cases
Plate 16. Worm decorating with silicone in lab

Plate 17. Microscope photograph silicone tube top
Tube top was broken up and photographed under microscope. Glue is apparent on individual pieces.

**Plate 18. Adhesive on silicone**

![Adhesive on silicone](image1)

**Plate 19. Adhesive encircling silicone**

![Adhesive encircling silicone](image2)

**Plate 20. Worm decorating with D4-silicone in lab**
Red glue can be observed on the outside of the silicone pieces.

**Plate 21. D4 infused silicone tube top under microscope**

D4-infused silicone tube top under microscope.
The worm adhered two pieces of D4-infused silicone to one another. The two pieces stuck to one another were pulled off of the tube, photographed, separated, and photographed again.

**Plate 25. D4-Silicone adhered to D4 Silicone**

![Image of adhered D4-Silicone](image)

**Plate 26. D4-Silicone adhered to D4 Silicone**

![Image of adhered D4-Silicone](image)

Adhesive encircles the silicone.
Adhesive is on the face of the silicone.

**Plate 27. Silicone infused D5**

The worm only decorated with one piece of silicone infused with D5.

**Plate 28. Sand**
Antifouling Substrates

The volume of the control, D4, and D5 infused silicone pieces was calculated prior and post-infusion. The diameter and average of the left, center, and middle height was measured to calculate the volume of each piece where Volume=$\pi$(radius$^2$)(height).

Plate 31. Silicone prior and post to D4 infusion

The height of silicone prior (A) and post (B) D4 infusion.
The swelling from infusion is visible by viewing pre and post-infusion photographs. The volume of each piece of D4 infused silicone was calculated prior and post infusion. The volume prior to infusion is about $34.969\, \text{mm}^3$ and $35.169\, \text{mm}^3$ post infusion.

**Figure 3. Volume of silicone prior and post D4 infusion**

Bars are mean volume. Error bars are standard error of the mean. (n=30)

The change in volume of individual pieces post D4 infusion minus pre-infusion is plotted below (n=30). Half of the pieces increased in volume and the other half decreased.
The volume of silicone pieces prior and post D5 infusion was calculated. The volume prior to infusion is about 42.76 mm$^3$ and 35.173 mm$^3$ post infusion.

**Figure 5. Volume of silicone prior and post D5 infusion**

Bars are mean volume. Error bars are standard error of the mean. (n=30)

The change in volume of pieces prior to D5 infusion minus post-infusion volume shows that 22 out of 30 pieces decreased in volume while 8 increased.
Antifouling Substrate Decoration

After 24 hours, nine worms decorated with varying number of silicone and shell pieces.

Only one worm out of ten total did not decorate. Worms used about 7 D4 and D5 pieces and only about 1.5 shell pieces on average.

Figure 5. Number of Decorations Used
Bars are mean decorations used. Error bars are standard error of the mean. (n=12 worms total, n=120 decorations)

**Observation of Glue on Silicones**

The surface area of glue was measured on each of the decoration types: silicone, D4 infused silicone, D5 infused silicone, and natural shell. D4 decorations had the most adhesive and shell had the least. N=30 for each decoration type.

**Figure 6. Glue Surface Area by Decoration**
The bars show the mean surface area of adhesive in mm$^2$. Error bars are standard error of the mean.

Glue was examined qualitatively under a microscope. I deduced four categories of glue based on observation: brown masses, black strings, red masses, and red strings.

**Plate 13. Brown masses of adhesive**

This is an example of the brown masses of adhesive.

**Plate 15. Red mass of adhesive cool**

Red masses of glue found only on silicone and D4 infused silicone.
Plate 16. Red string of adhesive

Red string adhesive is in the bottom right corner. Red string adhesive was found only on silicone and D4 groups.

The majority of adhesive was brown masses (Plate 13), which comprised 100% of adhesive on shell, 94% of adhesive on silicone, 73% of adhesive on D4, and 100% of adhesive on D5. Figure 7 shows the percentage of glues that appeared as red mass and red strings. These only appeared on silicone and D4 pieces. Red masses comprised about 4% of glue on silicone and 14% on D4. Red string comprised about 2% on silicone and 13% on D4.

Figure 7. Percentage of glue type surface area by decoration type
The percentage of surface area of each glue type in terms of shell decorations, silicone, D4 infused silicone, and D5 infused silicone adhered to the tube.

The glue surface area was measured after measuring the adhesive strength using the pulley system.

**Plate 17. Pulley system**
Shell had an adhesive strength a great deal stronger than the other substrates.

**Adhesive Strength: Tube decorations**

<table>
<thead>
<tr>
<th>Adhesive Strength (MPa)/mm²</th>
<th>Shell</th>
<th>Silicone</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesive Strength (MPa)/mm²</td>
<td>0.005</td>
<td>0.004</td>
<td>0.003</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Shell had an adhesive strength a great deal stronger than the other substrates.

**Adhesive Strength: Silicone adhered to silicone**

The adhesion strength of two or more silicone pieces glued to one another (silicone, D4, and D4 group) was greater than the adhesion strength of the tube decorations. When measuring adhesive strength, the Gorilla Glue (that attached the silicone pieces to the pulley system) unattached before the two pieces could be pulled apart; thus, the adhesive strength of the silicone adhered to one another was stronger than Gorilla Glue adhered to
the silicone and D5 groups. D4 adhered to D4 adhesive strength was measured because
the Gorilla Glue didn’t unattach from the silicone, allowing weight to be added to the
pulley system. The adhesive strength between the two groups of two D4 pieces was
1.424669604 MPa/mm² and 4.04745167MPa/mm². The strongest adhesive strength of
silicone decorations glued to the tube was D5 infused silicone, Group 2 with an adhesive
strength of 0.000736176 MPa/mm². The strongest adhesive strength of decorations was
that of natural shell decorations in Group 3 at 0.005092913 MPa/mm², which was still
significantly less than the adhesive strength of two silicone pieces adhered to one another.
In three of the decorations adhered to one another, the Gorilla Glue did not detach.
However, the adhesive strength could still not be measured because the pulley was
holding the maximum weight possible of 300 grams. The adhesion force was at the
minimum 2.94 N for those decorations.

Discussion

When the tube top is removed, decorator worms, as is the case for sandcastle worms,
compulsively rebuild tubes (Stewart et al., 2011). D. cuprea push or pull nearby
decorations to the tube and rub the substrate with the branchiae, possibly to remove
biofilms (Stewart, 2011) or add glue. Rubbing behavior has been observed in mussels
(Waite, 1992, Stewart, 2011) and sandcastle worms (Stevens et al., 2007, Stewart, 2011).
We made worms rebuild tubes with glass beads and used the beads and tube in wet
chemistry experiments. Decorator worm adhesive showed high protein presence from
Coomassie Blue staining and high phosphate presence, especially in the tube (as opposed
to glue on glass beads) from the Phosphate Colorimetric experiment.
Preliminary results show similarities between *D. cuprea* and *Phragmatopoma californica* adhesive. Sandcastle worm *P. californica* bioadhesive contains at least 6 oppositely charged proteins that use phosphoserines as an underwater adhesive promoter (Wang & Stewart, 2013). Coomassie blue staining shows high protein presence in Decorator worm bioadhesive. Alcian blue staining of *P. californica* adhesive showed low abundance of glycosylated proteins (Wang & Stewart, 2013) much like Alcian blue staining in *D. cuprea*. Periwinkle Snail trail mucus is carbohydrate-rich with small amounts of proteins. The snail adhesive had 2.7 more proteins than its trail mucus (Smith & Morin, 2002). Carbohydrate adhesives are rich in glycoproteins (Emengo *et al*., 2002) but glycoproteins aren’t primary adhesive components for marine invertebrates.

Proteinaceous underwater adhesives are common in aquatic invertebrates. Mussels, sandcastle worms, caddisflies, sea cucumbers, and midge larvae use glues with post-translational modifications (PTM) such as phosphates on amino acids (Stewart, 2011). Though barnacle glue is reported to not contain post-translational modifications (review; Stewart, 2011, Kamino, 2010), recent studies find phosphorylated proteins in glues (Dickinson *et al*., in press, Gohad *et al*., 2014). Phosphorylated proteins, like those found in the Decorator worm adhesive, may be a conserved underwater adhesive mechanism across taxa (Stewart, 2011) due to the presence of phosphorylated serines in mussels (Waite and Qin 2001; Stewart, 2011), sandcastle worm (Waite *et al*., 1992; Zhao *et al*., 2005; Stewart *et al*., 2004; Stewart, 2011), caddisfly silk (Stewart and Wang 2010; Stewart, 2011), midge larva (Kao and Case, 1985; Stewart, 2011), and sea cucumber tube (Flammang *et al*., 2009; Stewart, 2011). Although phosphoserines specifically have not yet been tested in Decorator worm adhesive, the quantification of phosphoprotein
presence in this worm’s adhesive may further suggest that phosphates are a conserved molecular mechanism in underwater adhesion.

The decorator worm utilizes its underwater adhesive to create a microreef on the outside of its tube to attract prey (Mangum et al., 1968; Woodin 1978). In addition to the ecological importance of the adhesive in enabling the worm to use its tube as food-catching tool, the worm’s tube-building behavior allowed us to use the worm as a new model system for testing foul-release materials. In the lab, the worm decorated with any of the materials given. These materials could be tested further in order to understand how natural underwater adhesives interact with antifouling substrates. Capillary action, greater amount of adhesive, or altered standard adhesive may have resulted in greater adhesive strength between silicone glued to one another.

*D. cuprea* are reported to be up to 30 cm in body length with tube lengths up to 1 m and tube caps at about 5 cm (Woodin, 1978). *D. cuprea* used in this study were about 8 cm. Common bioadhesive polychaete models *Phragmatopoma californica* and *Hydroides elegans* are up to 7.5 cm (Hinton, 1988) and 2-8 centimeters in length (Imajima, 1976; Masterson, 2007). Although these worms are beneficial for studying bioadhesive in colonies and fouling polychaetes respectively, the large size of *D. cuprea* is a beneficial model to the great amounts of adhesive found on a large decoration surface. The large amounts of bioadhesive and propensity of the worm to use any material in the correct size range to rapidly decorate their tubes decreases the time needed to test antifouling materials.

*D. cuprea* cured adhesive is easily harvested since it adheres materials to the external tube. For instance, the worm decorated with silicone loaded with cyclic siloxanes found
in foul-release coatings that have been shown to alter the enzyme activity of barnacle bioadhesive (Rittschof, et al., 2011). Worms decorated with modified, charged glass that may interfere with adhesive strength due to polybasic proteins in bioadhesive. The large worms readily adhere any peripheral decoration making them an excellent candidate for studying bioadhesive response to antifouling substrates and histological or biochemical studies of glue production.

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Citations


