BARNACLE CEMENT:
A POLYMERIZATION MODEL BASED ON EVOLUTIONARY CONCEPTS

by

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Duke University

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in
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ABSTRACT

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Abstract

The tenacity by which barnacles adhere has sparked a long history of scientific investigation into their adhesive mechanisms. To adhere, barnacles utilize proteinaceous cement that rapidly polymerizes and forms adhesive bonds underwater, and is insoluble once polymerized. Although progress has been made towards understanding the chemical properties of cement proteins, the biochemical mechanisms of cement polymerization remain largely unknown. In this dissertation, I used evolutionary concepts to elucidate barnacle cement polymerization. Well-studied biological phenomena (blood coagulation in vertebrates and invertebrates) were used as models to generate hypotheses on proteins/biochemical mechanisms involved in cement polymerization. These model systems are under similar selective pressures to cement polymerization (life or death situations) and show similar chemical characteristics (soluble protein that quickly/efficiently coagulates).

I describe a novel method for collection of unpolymerized cement. Multiple, independent techniques (AFM, FTIR, chemical staining for peroxidase and tandem mass spectroscopy) support the validity of the collection technique. Identification of a large number of proteins besides ‘barnacle cement proteins’ with mass spectrometry, and observations of hemocytes in unpolymerized cement inspired the hypothesis that barnacle cement is hemolymph.

A striking biochemical resemblance was shown between barnacle cement polymerization and vertebrate blood coagulation. Clotted fibrin and polymerized cement were shown to be structurally similar (mesh of fibrous protein) but biochemically distinct. Heparin, trypsin inhibitor and Ca$^{2+}$ chelators impeded cement polymerization, suggesting trypsin and Ca$^{2+}$ involvement in polymerization. The presence/activity of a
cement trypsin-like serine protease was verified and shown homologous to bovine pancreatic trypsin. Protease activity may activate cement structural precursors, allowing loose assembly with other structural proteins and surface rearrangement. Tandem mass spectrometry and Western blotting revealed a homologous protein to human coagulation factor XIII (fibrin stabilizing factor: transglutaminase that covalently cross-links fibrin monomers). Transglutaminase activity was verified and may covalently cross-link assembled cement monomers.

Similar to other protein coagulation systems, heritable defects occur during cement polymerization. High plasma protein concentration combined with sub-optimal enzyme, and/or cofactor concentrations and sub-optimal physical/muscular parameters (associated with hemolymph release) results in improperly cured cement in certain individuals when polymerization occurs in contact with low surface energy silicone and its associated leached molecules.
Dedication

This work is dedicated to my parents,

my fiancé Cindy, my brother Andy, and my sister Jen,

who have encouraged, inspired and supported me throughout my Ph.D.
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1. Introduction

Since Darwin’s definitive monographs on the Cirripedia (Lepadidae, 1851; Balanidae and Verrucidae, 1854), barnacles have been the subject of notable scientific research. Much of this research has focused on barnacle diversity, phylogeny and evolution (reviewed in Schram and Hoeg 1995), as well as ecology, larval behavior and physiology (reviewed in Southward 1987, Anderson 1994). Within the past 40 years, the tenacity by which barnacles adhere has sparked scientific investigation into their adhesive mechanisms. To adhere, barnacles utilize a proteinaceous cement that rapidly polymerizes and forms adhesive bonds underwater, and is insoluble once polymerized (reviewed in Kamino 2006, Kamino 2008). Research interest into barnacle adhesive mechanisms lies primarily in the development of marine coatings that diminish barnacle adhesive strength, since fouling of ship hulls by barnacles and other organisms cost the defense and shipping industries billions of dollars every year. The ability of barnacle cement to quickly and effectively polymerize underwater has also lead researchers to consider medical and dental applications of isolated cement components (Carderelli 1968, Kaplan et al. 2003).

Barnacle cement has traditionally been studied in isolation from other biological processes through amino acid sequence analyses of isolated cement proteins. Although progress has been made towards understanding the chemical properties of specific cement proteins using these techniques (reviewed in Kamino 2006, Kamino 2008), the biochemical mechanisms by which cement polymerization occurs remain largely unknown. Basic biochemical investigations into the nature of barnacle cement have been hindered by the inherent insolubility of polymerized cement. Creative techniques developed to obtain cement prior to complete polymerization (Walker 1972, Cheung et
al. 1977), and partially denature polymerized cement (Barnes and Blackstock 1976, Yan and Pan 1981, Naldrett 1993, Kamino et al. 1996, Kamino 2001), have allowed for compositional analysis. Barnacle cement is composed of 90% protein (Walker 1972, Naldrett 1993) and is an aggregate of at least ten major proteins. Some, but not all, of the barnacle cement proteins have been isolated and sequenced (Kamino 2006, 2008). Based on the abundance of cystine in certain cement proteins and the ability to partially dissolve polymerized cement in reducing solvents, chemical stability of polymerized barnacle cement is thought to be achieved in part through cysteine cross-links and hydrophobic interactions (Barnes and Blackstock 1976, Naldrett and Kaplan 1997, Kamino et al. 2000).

In this dissertation I have taken a novel approach to elucidating the biochemical mechanisms involved in barnacle cement polymerization, unique from the traditional protein isolation and amino acid sequencing that has been conducted on barnacle cement in the past. I have applied evolutionary concepts to develop specific hypotheses on proteins and biochemical mechanisms that may be involved in cement polymerization. Specific evolutionary concepts include the tendency for proteins and biochemical mechanisms that work well to be conserved over evolutionary time and the concept of evolution as a gradual process involving minor modifications over time. Based on these concepts, similar proteins and biochemical mechanisms may be expected between systems that exhibit both similar selective pressures and similar general chemical properties. I have selected well-studied biological phenomena, vertebrate and invertebrate blood coagulation, as a model in which to study barnacle cement polymerization. These systems share selective pressures and general chemical properties with barnacle cement polymerization; blood coagulation and barnacle cement polymerization are both life or death situations in which soluble protein must quickly and efficiently coagulate.
1.1 Model System: Blood Coagulation

Blood coagulation is an ideal model system in which to consider barnacle cement polymerization, as the biochemical mechanism of blood coagulation has been well studied in vertebrates and invertebrates. In all vertebrates, formation of a stable fibrin centered blood clot is dependent on both proteolytic activation of fibrinogen, which allows electrostatic interaction of fibrin monomers (Doolittle 1984, Weisel 1986, Weisel et al. 1999, Sit and Marchant 2001), and covalent cross-linking of fibrin monomers by a proteolytically-activated transglutaminase (factor XIII: Lorand et al. 1962; Lorand et al. 1964). As shown in Figure 1, amplification of a stimulus (vascular injury) into a physiological response (a stable fibrin clot) is made possible by two closely interrelated cascades of trypsin-like serine proteases (Davie and Rantoff 1964, MacFarlane 1964). Components of the cascades are present in blood plasma in an inactive form and are sequentially converted to their active form by limited proteolysis. The intrinsic pathway consists of a series of reactions that utilize only factors present in blood plasma, and is initiated through contact with a negatively charged surface (reviewed in Davie and Fujikawa 1975, Davie 2003). Plasma components in addition to tissue factor (a cell surface expressed lipoprotein, released upon vascular injury) are required for the extrinsic pathway, which initiates overall clot formation. The activity of several of the blood coagulation factors is dependent on Ca$^{2+}$, as well as other non-enzymatic cofactors (phospholipid, protein cofactors VIII and V, and tissue factor).
Figure 1: The vertebrate blood coagulation cascade (simplified form). The cascade allows for the amplification of a stimulus (vascular injury) into a physiological response (a covalently cross-linked fibrin clot at the site of injury). Formation of a stable fibrin clot is dependent on both trypsin-like serine protease and transglutaminase activity. Blue are trypsin-like serine proteases in the inactive form, red activated trypsin-like serine proteases, light green inactive and dark green activated transglutaminase, and grey are essential cofactors. PL = phospholipid.
Blood coagulation in invertebrates occurs through a biochemically similar process to that in vertebrates, although the terminal substrate (coagulogen in horseshoe crabs, clotting protein in crustaceans) is biochemically distinct from vertebrate fibrinogen (Fuller and Doolittle 1971a, Ravindranath 1980, Doolittle 1987). Blood coagulation in horseshoe crabs occurs through two converging cascades of trypsin-like serine proteases, contained within blood cells as zymogens and released upon exposure to minute quantities of pathogens (reviewed in Muta and Iwanaga 1996, Sritunyalucksana and Soderhall 2000, Osaki and Kawabata 2004, Theopold et al. 2004). The proteolytic cascades involved in horseshoe crab blood coagulation are thought to be derived from an ancestral proteolytic cascade common to both horseshoe crab and vertebrate blood coagulation (Krem and Di Cera 2002). Blood coagulation in crustaceans occurs through the action of a Ca$^{2+}$ activated transglutaminase (Fuller and Doolittle 1971b, Lorand 1972, Kopacek et al. 1993) homologous to vertebrate factor XIIIa (Wang et al. 2001). Transglutaminase activity mediates covalent cross-linking of clotting protein. Trypsin-like serine protease activity has been shown in the crustacean blood coagulation process, and may be involved in proteolytic activation of structural proteins (allowing loose assembly prior to transglutaminase mediated cross-linking; Madaras 1981; Durliat and Vranckx 1981) and activation of the pro-phenoloxidase pathway (Soderhall 1981).
1.2 Alternative Cement Morphology on Silicone

Studies of defective blood coagulation have been key to elucidating the biochemical mechanisms of vertebrate blood coagulation (Davie 2003). Mixing and complementation experiments using blood from patients with blood clotting disorders have enabled identification of specific clotting factors. Genetic variability in the morphology of polymerized barnacle cement has been documented (Holm et al. 2005). Altered cement morphology may result from specific differences in the cement protein polymerization process, similar to those observed in blood coagulation. Variability in cement morphology is exclusively expressed when barnacles are grown on low surface energy silicone coatings. On high surface energy substrates (such as glass or polystyrene), barnacles always produce a thin, hard, transparent cement (Figure 2A). In contrast, barnacles grown on low surface energy silicone coatings occasionally produce a thick, gummy, opaque cement, which may be improperly cured (Figure 2B). Gummy cement is noticeably soft and can be several millimeters thick. For Amphibalanus amphitrite (= Balanus amphitrite) (Pitombo 2004) the proportion of individuals producing gummy cement varies with the type of silicone substrate, with 31% of individuals showing gummy cement on Veridian® silicone, a commercial mold-release coating, and 18% exhibiting this trait on Silastic T2®, a silicone rubber (Holm et al. 2005). Throughout this document barnacles expressing the hard, thin cement phenotype (Figure 2A) will be referred to as “hard” and barnacles expressing thick, opaque, gummy cement (Figure 2B) will be referred to as “gummy”.
At present, is it unknown if underlying differences in cement chemistry account for the expression of hard versus gummy cement, or if these alternative phenotypes arise simply from a differing physical response to a low surface energy substrate. This question will be the focus of Chapter 4. Given the potential for differences in cement chemistry between hard and gummy barnacles, throughout this dissertation the use of hard or gummy barnacles will be explicitly stated.

Figure 2: The base plate of a hard barnacle (A) and a gummy barnacle (B), both *Amphibalanus amphitrite*. Barnacles were grown on a Veridian® silicone coated glass plate. Note the radial canals of the base plate, which are clearly seen for the hard barnacle but cannot be seen for the gummy barnacle.
1.3 Objectives

The overall objective of this dissertation is to develop a model for the polymerization of barnacle cement. Vertebrate and invertebrate blood coagulation, well studied biological phenomena with similar chemical characteristics (rapid and efficient coagulation of soluble protein) and similar selective pressures (life or death situations) to barnacle cement polymerization will be used as models for the development of specific hypotheses on cement polymerization. The data presented in three chapters, written in manuscript form, will be integrated in a cement polymerization model presented in the final chapter of this dissertation. Briefly, Chapter 1 will assess the ability to collect cement in the unpolymerized state and the involvement of hemolymph in cement polymerization, Chapter 2 will consider if barnacle cement polymerization and vertebrate blood coagulation occur by a similar enzymatic mechanism and determine if these systems share homologous proteins, and Chapter 3 will evaluate if a chemical basis for the expression of hard versus gummy cement exists and determine if specific defects in the cement polymerization process result in the formation of gummy, improperly cured cement. The cement polymerization model developed in this dissertation will be broadly relevant to the management of biological fouling (through the identification of specific inhibitors of cement polymerization) and the creation of biomimetic adhesives that cure underwater or in vitro.
2. Barnacle Hemolymph Functions as Cement

2.1 Introduction

The fitness of sessile organisms is dependent on a reliable attachment mechanism. For marine organisms that permanently attach to hard substrates, attachment is typically derived from a secreted adhesive with specific chemical properties. These adhesives are able to displace water, spread and form adhesive bonds with the substrate as well as coagulate/cross-link, which imparts stability to the adhesive (Waite 1987). To date, marine mussels are the only organisms in which adhesive biochemical composition and cross-linking is well understood. Marine mussel adhesive is composed of proteins with extensive post-translational modifications, occurring in highly repetitive sequences motifs (Waite and Tanzer 1981, Waite 1983, Waite et al. 1985, Filpula et al. 1990). Hydroxylated and charged amino acids comprise the majority of adhesive proteins, allowing for surface interactions. Cross-linking of the cement is brought about by the oxidation of DOPA (dihydroxyphenylalanine) residues, which are chelated by oxidizing metals such as Fe$^{3+}$ (Monahan and Wilker 2004, Sever et al. 2004).

Barnacles are one of the most common and dominant members of marine fouling communities, which has spurred research into their adhesive mechanisms. As compared to marine mussels, detailed biochemical knowledge of cement properties and their cross-linking mechanism is lacking. Basic biochemical investigations into the nature of barnacle cement have been thwarted by its inherent insolubility. Polymerized barnacle cement has not been rendered fully soluble under any conditions (Kamino 2006). Barnacle cement is composed of approximately 90% protein (Walker 1972, Naldrett 1993, Kamino et al. 2000). The remaining 10% consists of trace levels of carbohydrate (1%), lipid (1%) and
inorganic ash (4%) with 30% of the inorganic ash as calcium (Walker 1972). Barnacle cement is an aggregate of at least ten major proteins, some (but not all) of which have been isolated and sequenced (reviewed in Kamino 2006, 2008). The extensive post-translational modifications and repetitive sequences observed for mussel adhesive proteins have not been shown in barnacle cement (Naldrett 1993, Naldrett and Kaplan 1997, Urushida et al. 2007), indicating that the polymerization mechanism in these two organisms is fundamentally different. In part, chemical stability of polymerized barnacle cement is achieved through cysteine cross-links and hydrophobic interactions (Barnes and Blackstock 1976, Naldrett and Kaplan 1997, Kamino et al. 2000).

Acquisition of barnacle cement prior to polymerization is essential for the study of cement polymerization mechanisms and for complete compositional analysis. Barnacle cement production is continuous throughout a barnacle’s life and hence collection of cement in the unpolymerized state should be possible (Saroyan et al. 1970). Techniques previously proposed for the collection of unpolymerized cement have involved removing the barnacle from a substrate and propping them so that the base does not come into contact with a surface (e.g. fitting barnacles into a “plasticine girdle” described by Walker 1972, or floating Petri dish described by Cheung et al. 1977). The volume of cement that can be collected utilizing these methods, however, is limited, cement can be contaminated by exposure to seawater, and collection depends on the barnacles releasing cement themselves. As polymerization of cement has been observed within 15 min (Saroyan et al. 1970; Chapter 4 of this document), collection of unpolymerized cement requires either constant vigilance of barnacle bases and immediate collection when cement is released, or acceptance of the use of partially polymerized cement. In either case comparative analyses among barnacles are impossible. I suggest that aiding the cement release process by removing previously polymerized/calcified cement from the cement ducts will facilitate the release of
unpolymerized cement. This suggestion is supported by barnacle reattachment studies (Rittshcof et al. 2008; Chapter 4, ATR time course) showing that forced removal of an intact barnacle from a substrate, which likely dislodges polymerized cement from cement ducts, results in rapid release of unpolymerized cement.

Barnacle cement is generally studied in isolation from other physiological processes, leading to a somewhat narrow view of the cement polymerization process. Cement synthesis occurs in histologically distinct cement glands (Lacombe and Liguori 1969, Lacombe 1970, Walker 1970, Fyhn and Costlow 1976) and the synthesis of at least two of the cement proteins that have been isolated and sequenced from polymerized cement (19 and 100 kDa) has been localized in the basal region of the barnacle where the cement glands occur (as shown by Northern blotting, Kamino et al. 2000, Urushida et al. 2007). Synthesis within cement glands, however, does not indicate that these proteins are present only within cement glands nor does this indicate that other proteins are not involved in cementation. Insight may be gained into the cement polymerization process by broadening the view of this process to consider other processes in which polymerization of proteins occurs. One such process, hemolymph coagulation, has been well studied in crustaceans (reviewed in Durliat 1985, Sritunyalucksana and Soderhall 2000, Theopold et al. 2004), and although the biochemical mechanism has not been described in detail for barnacles, hemolymph has been shown to coagulate (Fitzgerald 1968, Waite and Walker 1988, Kamiya et al. 2002).

The primary objective of this study is to determine if unpolymerized barnacle cement can be collected in sufficient quantities to conduct biochemical assays. Multiple techniques (atomic force microscopy, infrared spectroscopy, chemical staining and tandem mass spectrometry) will be used to confirm the identity of collected secretions as barnacle cement. Secondarily, this study will consider if hemolymph components (proteins and blood cells) are contained within unpolymerized cement samples. The
potential role of these components in the polymerization process will be discussed in relation to crustacean blood coagulation.
2.2 Materials and Methods

2.2.1 Barnacle Larval Culture, Settlement and Maintenance

The barnacle *Amphibalanus amphitrite* (= *Balanus amphitrite*) (Pitombo 2004) was used for this study. Barnacle larval culture and settlement was conducted at the Duke University Marine Laboratory in Beaufort, North Carolina, following Rittschof et al. (1984a). Barnacle larvae were settled on 7.6 x 15.2 x 0.64 cm glass panels coated with silicone (Dow Corning Silastic T2® or International Veridian®) and maintained in the laboratory as described by Holm et al. (2005).

For experiments conducted at the Naval Research Laboratory (NRL), barnacles on silicone coated panels were transported to the Naval Research Laboratory in Washington, DC after 5 weeks of growth. While at NRL panels were kept in individual plastic containers filled with artificial seawater (32 ppt, Instant Ocean® in doubly distilled water, aerated overnight before use). Artificial seawater was changed twice a week. At NRL, barnacles were fed with 10 ml dense *Artemia* sp. (Sanders, Morgan, UT, hatched from approximately 1 teaspoon cysts in 1 L seawater) every day for 10 weeks and then every other day thereafter.

2.2.2 Unpolymerized Barnacle Cement Collection Method

Initial attempts at unpolymerized cement collection followed Cheung et al. (1977) directly. Barnacles were gently pried from treated wooden pilings, brought into the laboratory and suspended in an inverted position by fitting them through a hole (cut with a soldering iron) into a 100 x 15 mm polystyrene Petri dish. Petri dishes were floated in 20 cm diameter glass finger bowls containing ~ 1 L seawater (32 ppt). Although unpolymerized cement was observed, it was difficult to collect in large
quantities and the time of cement release was unknown. Therefore an alternative method, inspired by Cheung et al. (1977), was developed that incorporated the removal of previously polymerized/calcified cement from cement ducts.

For this alternative method, barnacles were gently removed from a silicone foul-release substrate using a dissecting needle (Hamilton Bell Co. Inc., Montvale, NJ). Barnacles whose base plate broke upon removal were discarded. Immediately following release from silicone, barnacles were classified by cement type (hard or gummy). Barnacles were considered “hard” if there was no opaque, soft cement on the base plate (as determined by visual inspection and gentle probing with a dissecting needle) and were considered “gummy” if >90% of the base plate was covered in opaque, soft cement. Barnacles showing intermediate levels of opaque cement coverage were not used. Only barnacles whose bases were completely in contact with the silicone panel were utilized for this study; barnacles growing in contact with other barnacles or on the edge of the panels were not used. Classification as hard or gummy was conducted immediately upon release since the appearance of soft cement changes as the cement is exposed to air (see Chapter 4).

Following classification, all shell plates (including the base plate) were gently cleaned in deionized water with a cotton swab. Barnacles were then dried with a Kimwipe® and sat in air on a paper towel for 3 hrs. Allowing time for the barnacles to dry is essential for the formation of defined cement droplets. To stimulate release of cement, the periphery of the base plate (where cement is normally released during growth; the junction between the base plate and parietal plates) was gently pricked in an outward direction with a dissecting needle. Opening the cement channels by removing previously polymerized/calcified cement allows for 1-2 μl droplets to form, which can be taken up with a 0.5 – 10 μl pipettor with a micro tip. Very gently squeezing the barnacle between the thumb and finger (compressing the base plate towards the
operculum) increased cement volume. After cement collection, unattached barnacles
were maintained in 10.5 cm glass finger bowls for up to two months, with hard and
gummy held in separate finger bowls. To prevent strong adhesion to the glass during this
time, each barnacle was pushed gently to a different location in its finger bowl daily.
Barnacles were used on average once per week for cement collection as described above.

2.2.3 Validation of Cement Collection Method

Four independent methods were used to verify that the substance collected from
the base of the barnacle was barnacle cement: 1) atomic force microscopy; 2) infrared
spectroscopy; 3) chemical staining for peroxidase; and 4) tandem mass spectrometry.

2.2.3.1 Analysis of Primary Cement, Secondary Cement and Cement Droplets Using
Atomic Force Microscopy

Imaging of barnacle cement was conducted using a Veeco Nanoman Atomic Force
Microscope (AFM: Digital Instruments, Dimension 3100) in tapping mode. All imaging
was conducted in air. Imaging in air is appropriate for hard barnacles due to the fact
that: 1) the cement is not highly hydrated; and 2) the cement-surface interface is a dry,
solid to solid interface. Imaging in seawater would have led to unnatural changes in
cement morphology and possible dissolution of mineralized crystals. Images were
extracted using WSxM V. 3.0 Beta 11.1 (Horcas et al. 2007).

Primary cement (original cement secreted by the barnacle) was imaged in situ on
the base of three separate hard barnacles. Barnacles were gently removed from a silicone
foul-release substrate using a dissecting needle and rinsed briefly in deionized water.
The shell plates were dried using a Kimwipe®. For imaging, each barnacle was affixed to
a microscope slide in an inverted, level position using sculpting putty.

Secondary cement, used by barnacles for repair and reattachment (Saroyan et al.
1970), was obtained by reattaching barnacles to a glass microscope slide. Hard
barnacles were gently removed from a silicone foul-release substrate using a dissecting needle, dried with a Kimwipe® and placed directly onto a glass microscope slide. Barnacles were allowed to reattach in air for 3 hrs and immersed in seawater thereafter. After 48 hrs of reattachment, barnacles were removed from the glass slides with a dissecting needle. Slides were washed lightly with deionized water and the residual cement from five separate barnacles was imaged.

For AFM of cement droplets, four 1 μl cement droplets (collected from hard barnacles) were deposited individually onto 75 x 25 mm glass microscope slides and the droplet was either: 1) immediately covered with a 10 x 30 x 1 mm (l x w x d) glass slide (cut to size with a diamond scribe, placed with approximately 5 mm hanging over the edge of the 75 x 25 mm slide for ease of removal) and placed in seawater to simulate the barnacle-cement-substrate interface; 2) placed in seawater uncovered; 3) immediately covered with 10 x 30 x 1 mm glass slide and left to cure in air; or 4) allowed to cure in air, uncovered. After 48 hrs, slides in seawater were removed from seawater, the cover slide was removed from the covered treatments and slides that had been in seawater were washed lightly in deionized water. Each droplet was imaged in at least 5 different regions within the droplet.

2.2.3.2 Analysis of Secondary Cement and Cement Droplets Using Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was performed using Attenuated Total Reflectance (ATR). FTIR was conducted on a Nicolet Magna-IR 750 Spectrometer with a DTGS KBr detector. Germanium ATR crystals (Harrick Model # EJ2122, 45°, 50 x 10 x 2 mm) were used for IR experiments. ATR crystals were reused up to three times, with each crystal cleaned thoroughly with ethanol between assays. The ATR crystal was mounted in a stainless steel Harrick Horizon® multiple reflection ATR
accessory with a water-tight gasket. Prior to placement of barnacles or droplets onto the ATR crystal, at least 15 background spectra of the clean ATR crystal were taken over the course of 30 min. All background spectra for each assay were averaged to serve as a baseline for spectral analysis.

**2.2.3.2.1 IR of Secondary Cement**

To obtain IR spectra of secondary barnacle cement, 5-7 hard barnacles were removed from a Silastic T2® silicone coated panel using a dissecting needle, the shell plates of the barnacles were dried with a Kimwipe® and barnacles were immediately placed on an ATR crystal, on which they would secrete cement. The usable surface area of the ATR crystal measured 50 x 8 mm, which allowed for placement of 5–7 barnacles, depending on barnacle size. Three IR assays with hard barnacles were conducted.

Spectra acquisition began immediately after placement of barnacles on the ATR crystal. Spectra were taken summing either 32 or 16 scans at a resolution of 8 wavenumbers. Spectra were taken every minute for the first 15 min after placement, every 3 min for the next 45 min and every 10 – 15 min for the next 8 hrs. Barnacles were left to reattach in air for up to 23 hrs or until no change was observed in spectra. After this time period, a small amount (1-2 ml) of artificial seawater was added to the trough of the ATR element. Enough seawater was added to cover the barnacle base plates, but not to cover the operculum. A glass microscope slide was placed over the trough of the ATR element to minimize evaporation. Spectra were acquired every 10 – 15 min for 4 hrs while the barnacles’ base plates were covered in seawater.

Of interest to this experiment are spectra of residual cement left by reattaching barnacles. After 4 hrs in seawater, the ATR element was removed from the spectrometer and barnacles were carefully removed from the ATR crystal using forceps. Seawater was poured off the Ge crystal and the crystal was rinsed with a small amount of deionized
water and gently blown dry with air. Cement left by reattaching barnacles could clearly be seen on the ATR crystal. The ATR element with residual cement was then placed back on the spectrometer. Spectra of residual cement were taken every 15 min for 1 hr or until no changes in spectra were observed.

2.2.3.2.2 IR of Cement Droplets

For IR of cement droplets, unpolymerized cement was obtained as previously described from hard barnacles and placed in three, 3 μl droplets onto a clean, referenced ATR crystal. Spectra of cement droplets in air were taken for 30 min or until no change in spectra was observed. After 30 min, 2 ml artificial seawater was added to the trough of the ATR crystal. After 1 hr in seawater the ATR element was removed from the spectrometer, seawater was poured off the Ge crystal and the crystal was rinsed with a small amount of deionized water and gently blown dry with air. Residual cement droplets could clearly be seen on the ATR crystal. The ATR element with residual cement droplets was placed back on the spectrometer. Spectra of residual droplets were taken every 15 min for 1 hr or until no changes between spectra were observed. Two cement droplet IR assays were conducted.

2.2.3.3 Localization of Peroxidase Activity

Peroxidase staining was first observed at the periphery of adult barnacles. The intensity of observed staining led me to further assess peroxidase activity in other cement associated regions of the barnacle. A TMB (3, 3’ , 5, 5’-tetramethylbenzidine) substrate kit (Vector Laboratories #SK-4400) was used to determine the presence and location of peroxidase activity in barnacle cyprids, cyprid “footprints”, juvenile barnacles, adult barnacles, primary cement, secondary cement, unpolymerized cement droplets and hemolymph droplets. Peroxidase staining was conducted a minimum of
two times for each. Kit components (buffer, stabilizer, TMB, and \( \text{H}_2\text{O}_2 \)) were mixed drop-wise with distilled water as instructed by the manufacturer. TMB yields a blue precipitate upon reaction with peroxidase. All samples were incubated at room temperature in TMB substrate for 5-10 min and then washed in deionized water, unless otherwise noted.

\textit{2.2.3.3.1 Cyprids}

Cyprids were placed directly into the TMB substrate, approximately 10 at a time, and then sieved from the substrate using a \( 165 \mu \text{m} \) filter. Cyprids that had been stored at 4\(^\circ\)C were brought to room temperature before staining. Stained cyprids were imaged on a compound microscope (Leitz Laborlux 12) at 125x magnification. Cyprids were incubated in TMB substrate for 25 min.

Cyprid “footprints” (used by the cyprid for temporary adhesion during the exploratory stage), were obtained on both clean PVDF membranes (no protein transferred) and PVDF membranes with transferred barnacle cement protein. Transfer of barnacle cement proteins onto PVDF membrane was conducted as follows. For SDS-PAGE, reducing sample buffer containing 10\% (w/v) SDS and 5\% (v/v) \( \beta_2\)-mercaptoethanol (Modified from Laemmli 1970) was added directly to 1.5 \( \mu \text{l} \) unpolymerized cement (collected as described previously) and samples were heated at 100\(^\circ\)C for 4 min. Reducing sample buffer was added in excess (80\% total volume rather than 50\%) to prevent polymerization of cement proteins. Samples were run on a 4-20\% gradient gel (Pierce Precise Precast Protein Gel, \#25244: 15 lane, 25\(\mu\text{l}\)) along with molecular weight markers (Novagen Trail Mix 10 – 225 kDa Protein Markers, \#70980-3) at 40 volts for 15 min and then at 100 volts for 1 hr. Following SDS-PAGE, proteins were transferred to a PVDF membrane (Millipore Immobilon \textsuperscript{P}\textsuperscript{\text{\textregistered}}, 0.2 \( \mu \text{m} \) pore size, \#ISEQ 081 00). Proteins were transferred at 4\(^\circ\)C at 15 volts overnight. Tris-Glycine
transfer buffer (pH 8.3) contained 15% methanol. Gels were stained with Coomassie Brilliant Blue R-250 (BioRad Electrophoresis grade, #161 0400) after blotting to assess protein transfer.

For both clean PVDF membranes (no transferred protein) and PVDF membranes with transferred barnacle cement protein, the membrane was cut to 4 x 6 cm, activated with 100% methanol and soaked in deionized water for at least 20 min prior to use. The membrane was then placed at the bottom of a shallow plastic dish (the lid from a Fisher brand pipette tip box, 8 x 12 x 2 cm (l x w x d), comprised of linear polyethylene) and approximately 500 newly metamorphosed (day 0) cyprids and 200 ml filtered, aged seawater was poured onto the membrane. Cyprids were allowed to explore the surface for 24 hrs at 27-28°C on a 12:12 hour light:dark cycle, after which time the membrane was washed thoroughly with deionized water to remove adhering cyprids and the membrane was stained with TMB substrate. Control PVDF membranes (clean PVDF incubated in seawater at 27-28°C for 24 hrs without cyprids) were also stained.

2.2.3.3.2 Juvenile Barnacles

Removal of juvenile barnacles from any surface is extremely difficult without breaking the shell plates. Therefore, to assess the presence of peroxidase activity on the base of juvenile barnacles, barnacles were settled on dialysis membranes (Spectrum Spectra/Por 240 x 240 mm Flat Sheet Membranes with 6-8000 MWCO, #132 677). Settlement on dialysis membranes allowed for TMB (MW = 240 Da) to access the base of the barnacle without requiring removal of the animal. To settle cyprids, the 240 x 240 mm dialysis membrane was cut in half and placed over a shallow 8 cm finger bowl, with the membrane slightly depressed into the bowl. Approximately 10 cyprids and 4 ml filtered, aged seawater was added to the depressed portion of the membrane. Settled barnacles were observed after 72 hrs (at 27-28°C on a 12:12 hour light:dark cycle),
though the percentage of cyprids that settled (20-25% over 72 hrs) was lower than that typically observed for silicone or polystyrene. After 2-3 days growth on dialysis membranes, the membrane was rinsed in deionized water and suspended over an 8 cm finger bowl with the barnacles inverted. 1 ml of TMB substrate was added to the non-barnacle side of the membrane for staining.

2.2.3.3.3 Adult Barnacles

Staining of adult barnacle base plates was conducted by placing whole barnacles (removed from either silicone or treated wooden pilings, cleaned with a Q-tip in deionized water) into a 3 cm finger bowl with 1 ml TMB substrate so that the base, but not the operculum, was covered in substrate.

Residual primary cement is often left by a barnacle after removal from a silicone substrate. Primary cement was stained directly on the silicone substrate by adding a 1 ml droplet of TMB substrate on top of the residual cement. Secondary cement was obtained by reattaching barnacles to a glass microscope slide as describes for AFM imaging (3 hrs in air followed by 45 hrs in seawater). After removing barnacles with a dissecting needle, slides were rinsed in deionized water and immersed in TMB substrate.

Unpolymerized cement droplets were obtained as described previously and placed in 0.5 μl droplets onto an activated PVDF membrane. Hemolymph droplets were obtained by inserting a syringe into the body (prosoma) of the animal, through the operculum. Droplets were placed on an activated PVDF membrane and the membrane was immediately incubated in TMB substrate.

2.2.3.4 Analysis of Cement Proteins Using Tandem Mass Spectrometry

Tandem mass spectrometry was used to identify Balanoid barnacle proteins (for inclusive genera see Pitombo 2004) present in unpolymerized barnacle cement. Mass
spectrometry was conducted at the Mass Spec Based Proteomics Facility at the University of Puerto Rico, Rio Piedras Campus. Peptides for analysis were produced through direct trypsin digestion of unseparated cement droplets (in solution) and trypsin digestion of bands isolated from an SDS-PAGE gel (in gel).

In solution trypsin digestion of unpolymerized barnacle cement was conducted by adding 1 μl unpolymerized cement directly to 100 μl 40 mM ammonium bicarbonate with 10% acetonitrile (ACN; mass spectrometry grade). Two, 1 μl cement samples were taken from each of three hard and three gummy individuals (12 samples total). Trypsin Gold (Promega #V5280), reconstituted to 1 μg μl⁻¹ in 50 mM acetic acid, was added immediately to each sample. 2.0 μg trypsin was used for samples from gummy individuals whereas 1.3 μg trypsin was used for samples from hard individuals to account for differences in cement protein quantity between hard and gummy barnacles (see Chapter 4). Samples were incubated at 37°C for 15 hrs, after which time ACN was added to 50% total volume and samples were shipped overnight to the University of Puerto Rico for mass spec analysis as described below.

For in gel digestion, unpolymerized cement from both hard and gummy barnacles was run on SDS-PAGE under reducing conditions (4-20% gradient gel with 1 μl cement per lane) as described previously. Gels were stained overnight with Coomassie Blue R-250 (0.25% Coomassie, 7.5% acetic acid, 5.0% methanol). Gels were shipped via overnight mail to the University of Puerto Rico during staining by placing each gel in a sealed plastic bag with ~50 ml Coomassie Blue stain. Once in Puerto Rico, gels were destained in 40% methanol.

Following initial destaining, individual protein bands detected by Coomassie Blue staining were carefully excised from the gel using a scalpel, destained using 100 mM ammonium bicarbonate:50% ACN, and then dehydrated in 100% ACN. After removal of ACN by speed-vacuum, the gel slice was re-hydrated in 40mM ammonium
bicarbonate and 10% ACN. Trypsin (1 μg) was added and incubated overnight (18 h) at
37°C. The tryptic peptides were eluted from the gel slice by incubating the slice in a
solution containing 50% ACN and 5% formic acid for 1 h at room temperature.

The tryptic-peptides from both in gel and in solution samples were loaded onto a
Surveyor® HPLC system and peptides were eluted, using a gradient of ACN (0%-80%) in
0.2% formic acid/H₂O, directly into the electro-spray ionization (ESI) source. The eluted
tryptic peptides were infused into a LTQ mass spectrometer (Thermo Fisher) for tandem
mass spectrometry analysis of the proteins of interest.

Tandem mass spectra were extracted by BioWorks® V. 3.2. Charge state
deconvolution and deisotoping were not performed. All MS/MS samples were analyzed
using Sequest® (ThermoFinnigan, San Jose, CA; V. 2.7). Sequest was set to search the
Balanoid barnacle database (NCBI non-redundant sub-database) assuming the digestion
enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da
and a parent ion tolerance of 2.0 Da. Only those identified peptides that pass selection
filters imposed on the database search were taken into consideration for protein
identification (Xcorr higher than 1.5 (+1), 2.0 (+2) or 2.5 (+3); Delta Score >0.1; 10 or
more b and y ions; MS2 intensity of >5x10⁻⁴, peptide probability >E x10⁻²).
Additionally, Scaffold® (V. Scaffold-01_07_00, Proteome Software Inc., Portland, OR)
was used to validate MS/MS based peptide and protein identifications. Peptide
identifications were accepted if they could be established at greater than 95.0%
probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein
identifications were accepted if they could be established at greater than 99.0%
probability and contained at least 2 identified peptides. Protein probabilities were
assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that
contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.2.4 Presence of Hemocytes in Cement Droplets

The presence of hemocytes in cement droplets was verified and quantified using a hemocytometer (American Optical Corporation, #1483). Heparin (Ammonium salt, porcine intestinal mucosa, Sigma #H 0880) was used as an anticoagulant to reduce the rate of cement polymerization. A 1 μl droplet of 1 mg ml⁻¹ heparin was first placed onto the counting grid. 1 μl cement was added to the heparin solution immediately after removal from the barnacle base and a cover slip was placed over the cement/heparin solution. The hemocytometer was placed on a compound microscope under phase contrast optics and photographed using a digital camera. Cell counts were made by reviewing these images on a computer monitor. Cells were classified as hyaline, granular or agglomerations based on morphology as described in Bauchau (1981) and Hose et al. (1990). Hyaline cells are generally round with a central, large round nucleus. Granulocytes are generally larger than hyaline cells (up to twice as large), have a small nucleus and multiple granular inclusions. Cells were scored as “agglomerations” when they appeared as a clumping of several smaller cells. Hemocytes from 6 different cement droplets (collected from hard barnacles) were counted.
2.3 Results

2.3.1 Cement Collection

When barnacles were suspended in floating Petri dishes as described by Cheung et al. (1977), cement droplets were observed to form and polymerize at the periphery of the barnacle and along cracks in the base plate within days (Figure 3A & B). Droplets became increasingly hard and opalescent over time, appearing to partially calcify. Although cement droplets were observed, it was not possible to collect droplets in sufficient quantities for biochemical assays or to pinpoint the time of their release.

In contrast, the method described in this chapter, which involved removing previously polymerized/calcified cement from cement ducts, permitted collection of unpolymerized cement in microliter quantities immediately upon release from the barnacle. When barnacles were taken out of water and the shell plates were allowed to dry in air for 3 hours, droplets were formed upon gentle outward picking of the base plate periphery with a dissecting needle (Figure 3C & D). Cement droplets averaged 1 μl in volume, but were observed up to 6 μl. Droplet volume was increased if the barnacle was squeezed gently, compressing the base plate towards the operculum. If shell plates were not given sufficient time to dry, droplets adhered to the moist shell and were difficult to take up with a pipettor. Droplets contained on average 10.3 μg ul⁻¹ protein, and evidence of protein polymerization was observed within 2 min for gummy barnacles and 15 min for hard barnacles as described in Chapter 4. Cement droplets did not readily dissolve in seawater; droplets that had been deposited onto a polystyrene Petri dish, immediately covered in seawater (32 ppt) and left for 1 week, were still clearly visible after seawater was removed (Figure 3E & F). Cement was successfully collected from most barnacles on most days. Barnacles were re-used successfully for cement
collection over the course of months. Barnacles very rarely died following the cement collection procedure.

Figure 3: Collection of unpolymerized cement. A & B) Barnacle supported in a floating Petri dish as described by Cheung et al. (1977). Cement droplets are observed at the periphery and along cracks in the base plate. C) Gentle outward pricking of the barnacle base plate periphery using a dissecting needle. D) An unpolymerized cement droplet on the barnacle base. E & F) Three, 0.5 μl cement droplets were collected, deposited on a polystyrene Petri dish in three separate locations, immediately covered in seawater (32 ppt) and left for 1 week: E) Cement droplets upon removal from seawater; F) The same cement droplets stained with Coomassie Blue.

2.3.2 Validation of Cement Collection Method

2.3.2.1 Analysis of Primary Cement, Secondary Cement and Cement Droplets: AFM

Primary cement (on the base of hard barnacles), secondary cement (left on glass by reattaching barnacles) and cement droplets cured in air and in seawater (covered and uncovered) were imaged using AFM. Distinct fibrous regions were observed in primary cement of all barnacles imaged (Figure 4A). Likewise, distinct fibrous regions were also observed consistently in secondary cement and a fibrous region was observed for the cement droplet cured under seawater while covered with a cover slide (Figure 4B & C).
In each case, fibers were 50 - 100 nm in diameter. In addition to fibrous structures, AFM also revealed globular domains and crystalline structures (see Chapter 3). Fibrous regions, however, were the dominant structural motif in primary and secondary cement and were observed in the cement droplet cured in seawater while covered. Fibrous regions were not observed for cement droplets cured uncovered in air or uncovered in seawater. Rather, these droplets appeared as an amorphous globular or crystalline feather-like structure. Fibrous regions also did not occur when a droplet was cured in air while covered, suggesting that removal of the cover slide did not cause the formation of fibrous regions.

![AFM images](image)

**Figure 4: AFM of barnacle cement (hard barnacles).** A) Primary cement imaged directly from the base of a barnacle. B) Secondary cement left by a barnacle reattached to a glass slide for 2 days. C) 1 μl droplet of barnacle cement (manually obtained as unpolymerized cement), cured for 2 days on a glass slide in seawater while covered with a glass cover slide.

### 2.3.2.2 Analysis of Secondary Cement and Cement Droplets: FTIR

FTIR spectra of residual barnacle cement left by whole, reattaching barnacles and spectra of cement droplets, obtained manually as unpolymerized cement and deposited on the ATR crystal are nearly identical in both the number and position of peaks (Figure 5). All major peaks show the same position, within the 8 cm⁻¹ resolution at which spectra were taken. For residual cement and cement droplets, the amide I peak is broad and is centered around 1638 cm⁻¹. Very minor differences do occur in peak intensity
relative to other peaks in the spectra (e.g. 3200 – 2800 & 1200 – 1000 cm⁻¹). It is noted, however, the volume of cement in whole barnacle assays could not be controlled and likely differed between whole barnacle and cement droplet assays. Environmental conditions (air temperature & humidity) also could not be controlled. FTIR spectra were consistent among replicate trials.

![FTIR spectrum](image)

Figure 5: FTIR of residual barnacle cement (left by hard barnacles reattaching to an ATR crystal in air, then in seawater) and cement droplets (obtained manually as unpolymerized cement from hard barnacles and cured on an ATR crystal in air, then in seawater). Significant peaks labeled in cm⁻¹.
2.3.2.3 Localization of Peroxidase Activity

Peroxidase activity, as shown by TMB staining, was observed in all exterior regions associated with cementation (Figure 6). Observations were consistent among replicate stainings. In the cyprid, peroxidase activity was observed on the 2nd segment of the antennules (located distally to the carapace) and on the adhesive organ as shown in figure 6A. The base of the thoracic limbs also stained on some cyprids. Cyprids left ~100 μm diameter, elliptical dots on PVDF membranes (Fig. 6B). These are likely temporary adhesive “footprints” enlarged by precipitating stain. The ~100 μm diameter, elliptical dots were observed on both clean PVDF membrane (no transferred protein) and PVDF membrane with transferred barnacle cement protein. Abundance and density of these dots was far greater on PVDF membrane with transferred barnacle cement protein than on PVDF membrane without cement protein. The ~100 μm diameter, elliptical dots observed when cyprids were present, were not observed for control PVDF membrane staining (incubated in seawater without cyprids), although ~10 μm dots were visible on both control and treatment PVDF membranes.

The perimeter of juvenile and adult barnacles, where cement is released upon growth, clearly stained (Fig. 6C & D). For adult barnacles, staining was also observed in small cracks in the base plate from which cement may be released. The majority of the calcified base plate, however, did not stain. Peroxidase staining was observed for primary cement left on silicone after removing a barnacle from silicone and for secondary cement left by barnacles reattached to glass (Fig. 6E & F). Dot blots on PVDF membrane of manually obtained cement droplets and hemolymph from the prosoma stained intensely for peroxidase activity (Fig. 6G & H).
Figure 6: Peroxidase staining using TMB substrate. Staining is highlighted by white arrows. A) Cyprid: AT = Antennules carrying adhesive organ (AO); CP = Carapace; TL = Thoracic limbs, B) ~100 μm elliptical dots (likely temporary adhesive “footprints”) left by cyprids on a PVDF membrane with transferred cement protein, C) Juvenile (2 day old) barnacle, stained through dialysis membrane, D) Adult barnacle, E) Primary cement left on silicone after removal of barnacle, F) Secondary cement left on glass after reattachment, G) Cement droplet from base; H) Hemolymph from prosoma.
2.3.2.4 Analysis of Cement Proteins Using Tandem Mass Spectrometry

At the time of analysis, six barnacle cement proteins had been fully sequenced and were available in the NCBI Protein Database (www.ncbi.nlm.nih.gov). At 99% identification probability, all six cement proteins were identified upon in solution protein digestion of unpolymerized cement droplets (Table 1). Five of the six proteins were identified with in gel digestion regardless of the molecular weight of the excised band. The presence of the 6th cement protein, “CB-1, cement protein cyanogens bromide peptide, from Megabalanus rosa” was identified in 90 and 170 kDa protein bands, but not in 17 and 65 kDa protein bands. Cement proteins were identified in both hard and gummy barnacles. As shown in table 1, a large number (up to 48) of Balanoid barnacle proteins in addition to the barnacle cement proteins were identified with ≥ 2 peptides (at 95% peptide probability) for both in solution and in gel digestions.
Table 1: Tandem mass spectrometry of trypsin digested unpolymerized barnacle cement. Number of unique, matching peptides to Balanoid barnacle proteins. HS and GS are hard and gummy in solution digestion. 17, 65, 90 & 170 kDa are in gel bands for a gummy lane. Previously sequenced cement proteins, available in the NCBI database at the time of analysis, are highlighted in yellow. Proteins from *Amphibalanus amphitrite* (= *Balanus amphitrite*), the species used to obtain unpolymerized cement, are shown in blue.

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2.3.3 Presence of Hemocytes in Cement Droplets

Hemocytes are clearly visible in cement droplets (Figure 7; collected from hard barnacles). Cell counts averaged 173 cells per μl cement (SEM ± 21.2), with the vast majority of cells occurring as hyaline cells (mean = 84.7% per trial, SEM ± 2.2). Granular cells (likely semi-granular) and agglomerations were much less common at 10.1% ± 1.5 and 8.3% ± 2.0 per trial, respectively (mean ± SEM).

Figure 7: Barnacle hemocytes in unpolymerized barnacle cement (collected from hard barnacles), under phase contrast optics. A) Low magnification, hyaline cells are primarily observed. B) Hyaline cell with large central nucleus. C) Semi-granular cell with small nucleus (larger cell). Small hyaline cell is present below the semi-granular cell. D) Agglomeration (grouping of several smaller cells).
2.4 **Discussion**

2.4.1 **Validation of Cement Collection Method**

Unpolymerized barnacle cement was successfully collected using a method inspired by Cheung et al. (1977). Unlike previously described collection methods, unpolymerized cement could be collected in microliter quantities enabling a wide-range of biochemical assays. Fitting the barnacles into a cumbersome “plasticine girdle” described by Walker (1972) or floating Petri dish (Cheung et al. 1977) proved unnecessary for cement collection. Barnacle cement collected in the manner described in this chapter contained a large amount of protein, polymerization was observed after 2 – 15 min (for gummy and hard barnacles respectively) and the cement was not readily soluble in seawater. AFM, FTIR, peroxidase staining and tandem mass spectrometry were used to validate the identity of the proteinaceous material collected from the base of the barnacle as barnacle cement.

Atomic force microscopy (AFM) allowed for *in situ* examination of barnacle cement ultrastructure, without the need for critical point drying or sputter coating as would be required for scanning electron microscopy. AFM of primary cement (original cement secreted by the barnacle) on the base of the barnacle itself and secondary cement (left by barnacles on glass slides during reattachment) showed the ultrastructure to be dominated by fibrous motifs, indicative of progressive cross-linking of the cement. Fibers were 50 – 100 nm in diameter and occurred in a tightly interlocking network. Ultrastructural observations are consistent with SEM and AFM studies of barnacle cement by Wiegemann and Watermann (2003) and Liedert and Kesel (2005) who showed a mesh of interlocking adhesive fibers in the base plate and residual cement of barnacles grown on PDMS (polydimethylsiloxane). Fibrous regions were also observed
when a cement droplet, obtained as described in this chapter, was placed on a glass slide, covered with a cover slide and cured in seawater. The presence of fibrous regions in a cured cement droplet indicates that the structural and enzymatic components required for progressive cross-linking of cement are indeed present in cement droplets collected as described herein.

IR spectroscopy can provide detailed information on molecular conformation and chemical bonding of surfaces and interfaces. This method was used to assess structural similarity between polymerized barnacle cement released directly by barnacles onto an ATR crystal and cured in seawater to that of cement droplets collected as described in this chapter, placed onto an ATR crystal and cured in seawater. IR spectra for residual cement and cement droplets are nearly identical in both the number and intensity of peaks with all major peaks showing the same position (within the 8 cm⁻¹ resolution at which spectra were taken). In both cases the amide I peak is broad and centered around 1636-1639 cm⁻¹. The presence of amide regions, characteristic of proteins (reviewed in Barth and Zscherp 2002), indicates that protein is present and able to interact with the surface (the ATR crystal) for both residual cement and cement droplets. Although IR cannot be used to unambiguously identify a protein, especially when a mix of proteins is present (as is the case for barnacle cement), IR can provide information on the secondary structure of the protein present (Jackson and Mantsch 1995, Barth and Zscherp 2002). For both residual cement and cement droplets the amide I peak is quite broad, suggestive of a mix of secondary structures (α-helix, β-sheet, β-turns, random coil) as opposed to a single dominant secondary structure.

In addition to AFM and IR spectroscopy, chemical staining for peroxidase activity was used to validate the unpolymerized cement collection method described in this chapter. Biochemical similarity in terms of enzymatic (or oxidative) activity as shown by peroxidase staining is expected among unpolymerized cement collected as
described herein, primary cement, secondary cement, and cement associated regions of the barnacle if the cement collection method is valid (all regions should either stain or not stain). Unpolymerized cement droplets stain intensely for peroxidase. Likewise, peroxidase activity was shown in all regions of the barnacle (and barnacle larvae) associated with cementation, including the base plate periphery of adult and juvenile barnacles (where cement is normally released), primary cement left after detaching barnacles, and residual secondary cement left by reattaching barnacles. Peroxidase staining was also observed for hemolymph droplets collected from the body of the barnacle. The ubiquity of peroxidase staining in cement associated regions suggests a potential role of peroxidase activity in the cement polymerization process, as will be described further in this chapter.

Tandem mass spectrometry was the most direct technique used to assess the validity of the cement collection method described herein. If the proteinaceous material collected from the base of the barnacle is in fact barnacle cement, then peptides generated by tryptic digestion of this material should match those of previously sequenced and reported barnacle cement proteins (at the time of analysis, six barnacle cement proteins had been fully sequenced and were available in the NCBI Protein Database). At the 99% probability level, all six previously sequenced barnacle cement proteins were identified in unpolymerized barnacle cement samples. Barnacle cement proteins were identified in whole cement samples (in solution) and in protein bands excised from an SDS-PAGE gel. All six sequenced barnacle cement proteins were identified in 90 and 170 kDa protein bands and five of six were identified in 17 and 65 kDa protein bands. The presence of proteins in multiple protein bands can be due to physical redundancy in the protein mixture or bioinformatics redundancy, where peptides are mapped to more than one protein (Bradshaw 2005). The large number of peptides identified for each cement protein and low sequence homology among 19, 20
and 100 kDa barnacle cement proteins (Kamino 2006) suggests that the presence of proteins in multiple protein bands is due to physical redundancy. Protein bands present in SDS-PAGE gels are likely to be an aggregate of multiple proteins. Proteolytic activity (as described in Chapter 3) and subsequent cross-linking may result in protein aggregates containing a mix of various proteins.

2.4.2 Barnacle Hemolymph as Cement

It is apparent that previously sequenced “cement proteins” are not the sole constituent of unpolymerized cement samples. A large number of proteins in addition to cement proteins was identified in unpolymerized cement samples by tandem mass spectrometry (up to 48 separate proteins with ≥ 2 matching peptides at 95% peptide probability). Many of these proteins (up to 12) had been sequenced in Amphibalanus amphitrite, the species from which cement was collected for this study. Of particular note is the settlement inducing protein complex (SIPC), an α2-macroglobulin-like protein, localized in the cuticle of barnacle nauplii and cyprids and in the shell, cuticle-lined organs and hemolymph of adult barnacles (Dreanno et al. 2006a, Dreanno et al. 2006b). As described further in Chapter 3, α2-macroglobulins function as protease substrates and inhibitors (Sottrup-Jensen 1989), are involved in innate immunity (Armstrong and Quigley 1999) and are related to complement cascade proteins C3, C4 and C5 (Sottrup-Jensen 1987). Other A. amphitrite proteins identified include BCS 1-6 (Okazaki and Shizuri 2000), which are thought to be expressed during and related to cyprid settlement, but are shown here to still be present in the adult, 70 kDa heat shock protein (Cheng et al. 2003), which is expressed in response to environmental conditions, a G protein-coupled receptor (Isoai et al. 1996), as well as other proteins related to the SIPC.

In addition to a large number of proteins not previously described as “cement protein”, hemocytes were observed in unpolymerized cement samples. In combination,
these observations force the conclusion that either: 1) unpolymerized cement samples contain hemolymph, which is unrelated to the cementation process; or alternatively, 2) unpolymerized cement is hemolymph and the cement proteins observed are simply hemolymph proteins. I suggest the latter, cement is hemolymph, based on: 1) Adhesive properties including polymerization and progressive cross-linking (as shown by AFM) is observed for unpolymerized cement samples. These properties are unlikely to be observed if samples are diluted with a large number of unrelated proteins and cellular components. 2) Adhesive properties of hemolymph proteins have previously been described (Patent application: Kaplan et al. 2003). N-terminal sequence of the 35 kDa hemolymph protein analyzed is identical to that of a 35 kDa base plate (cement) protein. 3) Primary and secondary cement left by barnacles share enzymatic/oxidative properties with hemolymph droplets as shown by chemical staining for peroxidase. 4) Barnacles are able to rapidly repair their base plate upon injury. Injury will undoubtedly result in the release of hemolymph from the base plate. Staining characteristics of repaired regions of the base plate are the same as those of the barnacle’s primary (original) cement (Saroyan et al. 1970). 5) Barnacles possess a successful hemolymph coagulation/mineralization mechanism (Fitzgerald 1968, Waite and Walker 1988, Kamiya et al. 2002). An ability to clot hemolymph is necessary to reduce loss of blood and decrease pathogen invasion during injury (Hall et al. 1999, Theopold et al. 2004). Therefore a hemolymph coagulation mechanism is likely to have been in place prior to the adaptation of cirripedes to a sessile lifestyle. Modification of the existing hemolymph coagulation mechanism to allow transport of hemolymph out of the body to the site of attachment, rather than evolution of a second unrelated coagulation mechanism specifically for cement, may have occurred.

Barnacle cement proteins are produced in cement glands located within the mantle of the animal among the ovarioles (Lacombe and Liguori 1969, Lacombe 1970,
Walker 1970, Fyhn and Costlow 1976). Synthesis of the 100 and 19 kDa barnacle cement proteins (obtained by partial dissolution of polymerized cement) occurs within the basal region of the animal where the cement glands are located (as shown by Northern blotting, Kamino et al. 2000, Urushida et al. 2007). The morphology of the cement glands, as well as their distribution varies among species (Lacombe 1970, Walker 1970). Histological staining has shown cement glands to be distinctly polar with a region of the gland associated with synthesis and storage and a separate region involved in secretion. The secretion from the cement glands is proteinaceous (Walker 1970) and extrusion is thought to be aided by muscle fibers in the surrounding connective tissue or elastic fibers encircling cement ducts (Lacombe 1970). Accumulation and synthesis within the cement glands is related to the molting cycle and is at maximum at stage B1 of the intermolt (Fyhn and Costlow 1976). Secretory products of the cement glands follow through a series of branching canals within the mantle leading to the radial and circular ducts of the base plate, where the secretion is dispersed to the periphery of the base (Lacombe 1970, Walker 1970). The cement ducts are generated by sequential invagination of mantle epithelium and are subsequently lined with chitin in some species.

The cement glands may be responsible for synthesis of not only cement proteins but also a variety of other hemolymph proteins. The interaction of the cement glands, the cement duct network, and the barnacle’s circulatory system is unclear, owing to the fact that these systems have historically been studied in isolation. The circulatory system of thoracican barnacles is unique among the Crustacea in that it follows a fairly well defined route through vessels constructed of condensed connective tissue (Burnett 1987). In both lepadomorph and balanomorph barnacles the distribution of blood vessels within the basal region of the barnacle (as described in Burnett 1972, 1975, 1977, 1987) is quite similar to the distribution of cement ducts (as described in Lacombe and Liguori 1969, Lacombe 1970). As living tissue, both the cement glands and the epithelial lining
of the cement ducts must be supplied with hemolymph. I suggest that the cement ducts are a modification of the barnacle’s circulatory system, allowing for hemolymph to be transported to the base plate and delivered externally. Supply of hemolymph from the body (prosoma) of the animal to the cement glands allows for mixing of cement gland secretions into the circulatory system. After mixing of cement gland secretions with hemolymph at the cement ducts, a portion of the hemolymph is transported through specialized circulatory canals (cement ducts) to the base of the barnacle where it is released by the barnacle, clots upon contact with a foreign surface and subsequently calcifies. Hemolymph not entering the cement ducts follows vessels through the mantle of the barnacle and back to the body. Detailed histological analysis of the interaction between cement ducts and the barnacle’s circulatory system would provide insight into barnacle adhesive mechanisms.

2.4.3 Hemocytes: Involvement in Cement Polymerization

Hemocytes play a critical role in the coagulation of crustacean blood (Ravindranath 1980, Bauchau 1981, Jiravanichpaisal et al. 2006). The observation of hemocytes in unpolymerized cement is logical within the hypothesis that cement is hemolymph. Two distinct cell types were identified in unpolymerized barnacle cement, hyaline and semi-granular cells, with hyaline cells comprising the vast majority of the cells present. Agglomerations (groupings of cells), which were likely agglutinated cells, were also observed. Cell counts (173 cells μl⁻¹) were significantly higher than that observed for Balanus hameri hemolymph (2.5 cells μl⁻¹: Waite and Walker 1988) but were consistent with observations on the hemolymph of Megabalanus rosa at 570 - 790 cells μl⁻¹ (Kamiya et al. 2002). Variation in cell counts among species is likely due to a combination of factors including environmental conditions and season, stage of the molting cycle, reproductive activity and exposure to pathogens and other stressors.
(reviewed in Bauchau 1981, Johansson et al. 2000). Granular/semi-granular cells were observed by both Waite and Walker (1988) and Kamiya et al. (2002), but as in this study, were considerably less common than hyaline cells.

Coagulation of hemolymph in crustaceans relies on both cellular and plasma factors (Fuller and Doolittle 1971b, Kopacek et al. 1993, Perazzolo et al. 2005). Coagulation occurs though the covalent cross linking of clottable protein by a Ca\textsuperscript{2+} activated transglutaminase (reviewed in Durliat 1985, Sritunyalucksana and Soderhall 2000). Clottable protein is present in cell-free plasma whereas transglutaminase is primarily released by hemocytes upon lysis. Release of transglutaminase by hemocytes, and subsequent activation by Ca\textsuperscript{2+} results in covalent cross-linking of clottable protein. Transglutaminase is thought to be primarily released by hyaline cells (Omori et al. 1989, Martin et al. 1991), although expression of transglutaminase has been shown in semi-granular and granular cells as well as in low levels in muscle tissue (Wang et al. 2001). The release of components of the prophenoloxidase system (contained in granular and semi-granular cells) will increase the rate of cell lysis and thereby increase the amount of transglutaminase released (Soderhall and Cerenius 1998). The abundance of hyaline cells in unpolymerized barnacle cement, which are capable of releasing transglutaminase upon lysis, suggests that this enzyme plays a key role in covalent cross-linking of cement components.

2.4.4 Peroxidase Activity: Involvement in Cement Polymerization

Peroxidase activity is a common theme in cement associated regions of barnacles. As shown by reaction to TMB substrate, unpolymerized cement droplets, hemolymph droplets (from the prosoma), the base plate periphery of adult and juvenile barnacles, primary and secondary cement left by barnacles, as well as cyprid antennules and temporary adhesive “footprints” all show distinct peroxidase activity. Peroxidase
activity may be involved in enzymatic or oxidative cross-linking of cement proteins and/or may play a defensive role against invading pathogens.

Observed peroxidase staining may be due to the presence of either an oxidative enzyme or an oxidizing metal (Dr. J. Wilker, Purdue University, Pers. Comm.). In marine mussels, the activity of oxidizing metals (specifically Fe$^{3+}$) has been shown to stabilize adhesive proteins by promoting oxidative cross-linking of DOPA residues (Monahan and Wilker 2004, Sever et al. 2004). Oxidative cross-linking of DOPA residues has likewise been suggested for barnacle cyprids (Walker 1971, Phang et al. 2006). Although DOPA residues have not been identified in adult barnacle cement (Naldrett 1993, Naldrett and Kaplan 1997, Urushida et al. 2007), oxidative activity may be involved in covalent cross-linking of cement monomers through cross-linking of cysteine residues. Cysteine residues are relatively abundant in cement proteins (Walker 1972, Naldrett 1993) and disulfide bonds are thought to contribute significantly to the stability of polymerized barnacle cement (Barnes and Blackstock 1976, Naldrett 1993, Kamino et al. 1996, Naldrett and Kaplan 1997, Kamino et al. 2000). Assessment of the role of oxidative activity in barnacle cement polymerization is currently underway through the use of compounds capable of blocking oxidizing activity.

In addition to oxidative cross-linking, peroxidase activity may play a defensive role during cement polymerization. If cement truly is hemolymph as suggested, and a portion of the barnacle’s circulatory system is open to the outside environment, then a mechanism to fend off invading pathogens must be in place. Peroxidases have been shown to play a defensive role in both plants and animals through oxidative killing of invading pathogens and production of antimicrobial agents (Everse et al. 1991). Within the context of crustacean wound healing, a 76 kDa peroxidase “peroxinectin” has been identified, first in the in the crayfish \textit{Pacifastacus leniusculus} (Johansson et al. 1995) and subsequently in the black tiger shrimp, \textit{Penaeus monodon} (Sritunyalucksana et al. 2001),
the white shrimp *Litopenaeus vannamei* (Liu et al. 2004), and the freshwater prawn *Macrobrachium rosenbergii* (Hsu et al. 2006) at a slightly different molecular weight. Cell adhesion (Johansson and Soderhall 1988), opsonization (Thornqvist et al. 1994), and encapsulation properties (Kobayashi et al. 1990) have also been shown for this protein. Peroxinectin is contained within granular and semi-granular hemocytes (Liang et al. 1992) and is released upon activation of the prophenoloxidase cascade (Holmblad and Soderhall 1999). A peroxidase, similar to peroxinectin, localized in cement associated regions of barnacles would provide both defensive and cell adhesion capabilities.

### 2.4.5 Conclusions

A novel method for the collection of unpolymerized barnacle cement is presented in this chapter, which enables collection of microliter quantities of unpolymerized cement. AFM, FTIR, chemical staining for peroxidase and tandem mass spectrometry confirmed that the proteinaceous material collected was barnacle cement. The presence of a large number of proteins besides “barnacle cement proteins” as well as hemocytes in unpolymerized cement led to the hypothesis that cement is hemolymph. Several lines of evidence support this hypothesis including similar staining properties between injured regions of the barnacle base and primary cement (Saroyan et al. 1970) and the ability for barnacle hemolymph to successfully clot (Fitzgerald 1968, Waite and Walker 1988, Kamiya et al. 2002).

Various changes accompanied the evolution of sessile Cirripedes from a free-living ascothoracid-like ancestor, including the development of a closed, high pressure circulatory system (Burnett 1987). Modification of the circulatory system to allow release of hemolymph at the attachment region may have occurred along with these changes. As suggested, the cement glands are associated with the circulatory system of the barnacle and may synthesize hemolymph proteins other than “cement proteins”.
Proteins that have previously been described as “cement proteins” (isolated from polymerized cement) may be a subset of the hemolymph proteins that are retained within the cement after polymerization, and are accessible to dissolution upon chemical treatment.

The release and coagulation of barnacle hemolymph is depicted schematically in figure 8. Hemolymph is typically released through cement ducts upon molting. Physiological changes associated with molting will allow for a small gap to form between the base and parietal plates, and upon muscular contraction hemolymph will be forced through cement ducts into this gap. Injury and reattachment of a barnacle will circumvent this process and release hemolymph directly from any opened vessels. Clottable protein(s) within the hemolymph will clot upon contact with a foreign substrate, with cross-linking of protein dependent on both plasma components (clotting protein) and cellular factors (transglutaminase, primarily in hyaline cells) as well as other enzymes (trypsin-like serine proteases) and cofactors described in Chapter 3. Oxidative cross-linking may be involved in stabilizing the clot. Having a circulatory system open to the outside environment necessitates an immune system capable of fending off pathogens. This role is potentially fulfilled by peroxidase activity, cells capable of phagocytosis (hyaline cells: Johansson et al. 2000), as well as multiple lectins, which have been described as a major component of barnacle hemolymph and are capable of agglutination, opsonization, non-self recognition and have been shown to be involved in the mineralization process (Kamiya and Ogata 1983, Muramoto et al. 1985, Muramoto et al. 1994, Kamiya et al. 2002).
Figure 8: Release and subsequent polymerization of cement as described in Chapter 2. Black arrows indicate release, grey arrows indicate involvement in polymerization and red arrows indicate involvement in the immune response.
3. Barnacle Cement Polymerization is Biochemically Similar to Blood Coagulation

3.1 Introduction

The process of coagulation is essential to a wide variety of biological phenomena. Biological coagulation occurs when macromolecules undergo a transformation from the liquid to the solid phase. Blood coagulation and barnacle cement polymerization are two such biological phenomena. In both of these systems the coagulation of proteins serves a key role in survival and reproduction. Coagulation of blood proteins in vertebrates and invertebrates reduces loss of blood and decreases pathogen invasion during injury (Davie and Fujikawa 1975, Muta and Iwanaga 1996, Hall et al. 1999, Theopold et al. 2004). Barnacle cement polymerization enables barnacles to adhere to a substrate after metamorphosis to the adult form where they are then able to feed, grow and reproduce (Walker 1971). Ineffective coagulation in both of these systems will lead to death or a failure to reproduce and therefore an effective system of coagulation is a necessity.

Blood coagulation is the quintessential example of a biological phenomena in which polymerization of proteins is critical to an animal’s survival; its mechanism has been well studied in both vertebrates and invertebrates. In all vertebrates, formation of a stable blood clot is brought about by two closely interrelated proteolytic cascades (Davie and Rantoff 1964, MacFarlane 1964). The overall product of these cascades is a network of fibrin monomers, covalently cross-linked by a transglutaminase (factor XIII). In the blood coagulation cascade, zymogens are converted to their active form by limited proteolysis and then in turn activate the next zymogen in the cascade. All of the major coagulation cascade enzymes are trypsin-like serine proteases, derived from pancreatic trypsin, which cleave on the carboxyl side of arginine residues (Neurath 1984, 1986,
Variability in the non-proteolytic domains of these enzymes allow for substrate and cofactor binding specificity (Patthy 1993, Neurath 1999).

Blood coagulation in invertebrates occurs in much the same fashion as in vertebrates. Studies with horseshoe crabs have shown their blood to coagulate through two converging cascades of trypsin-like serine proteases, contained within blood cells as zymogens, and released upon exposure to minute quantities of pathogens (reviewed in Muta and Iwanaga 1996, Sritunyalucksana and Soderhall 2000, Osaki and Kawabata 2004, Theopold et al. 2004). Blood coagulation in crustaceans occurs though the action of a Ca\(^{2+}\) activated transglutaminase (Fuller and Doolittle 1971b, Lorand 1972, Kopacek et al. 1993) homologous to vertebrate factor XIIIa (Wang et al. 2001). Involvement of trypsin-like serine proteases in the crustacean blood coagulation process has also been shown (Durliat and Vranckx 1981, Madaras et al. 1981, Soderhall 1981).

Polymerization of cement proteins is critical to a barnacle’s successful adhesion and hence their ability to survive and reproduce. To adhere, barnacles utilize a proteinaceous cement, which polymerizes underwater and is insoluble once polymerized (Walker 1972, Kamino 2006). Simple viscous adhesive forces do not adequately account for the adhesive strength shown by barnacles, indicating that polymerization of cement protein occurs (Dougherty 1990a, b). Basic biochemical investigations into the nature of barnacle cement have been thwarted by its inherent insolubility. Creative techniques developed to obtain liquid cement (Walker 1972, Cheung et al. 1977) and denature solidified cement (Barnes and Blackstock 1976, Yan and Pan 1981, Naldrett 1993, Kamino et al. 1996, Kamino 2001) have allowed for compositional analysis. Barnacle cement is composed of 90% protein (Walker 1972, Naldrett 1993) and is an aggregate of at least ten major proteins (Naldrett and Kaplan 1997, Kamino 2006). Some, but not all, of the barnacle cement proteins have been isolated and sequenced (reviewed in Kamino 2006, 2008). In part, chemical stability of polymerized barnacle cement is achieved
through cysteine cross-links and hydrophobic interactions (Barnes and Blackstock 1976, Naldrett and Kaplan 1997, Kamino et al. 2000). Reagents that disrupt cysteine cross-links and hydrophobic interactions, however, do not render cement fully soluble indicating that these mechanism do not fully account for the inherent insolubility of barnacle cement. An additional mechanism capable of generating covalent bonds between cement proteins, such as transglutaminase, may be involved.

The biochemical mechanism by which barnacle cement polymerizes is poorly understood. There is some evidence that proteolytic enzymes (Dougherty 1996, 1997) and salinity (Nakano et al. 2007) play a role in cement polymerization. The details of these mechanisms, however, have not been fully explored. The dependence of barnacles on the success of their cement suggests that selection pressure on adequate cement polymerization is intense.

The activity of proteolytic enzymes, such as those involved in vertebrate and invertebrate blood coagulation and possibly involved in barnacle cement polymerization, is ubiquitous to biological systems (Neurath and Walsh 1976, Neurath 1986, Krem and Di Cera 2002). The adaptability of these enzymes is shown by their diverse range of functions. The tasks of proteolytic enzymes range from simple digestive function in primitive organisms to complex physiological control in higher organisms (Neurath 1984, Krem and Di Cera 2002). Serine proteases are extremely wide spread, existing in prokaryotes to vertebrates (Kraut 1977). In addition to blood coagulation, proteolytic cascades of serine proteases comprise a variety of systems including the complement reaction, fibrinolysis, and dorsal-ventral patterning in drosophila (Neurath 1984, Krem and Di Cera 2002). The overall product of the proteolytic cascade in each system is the amplification of a small stimulus into a physiological response (Neurath and Walsh 1976, Neurath 1986). Proteolytic cascades are efficient and easily regulated with serine protease inhibitors, cofactors and specific feedback mechanisms.
Based on the evolutionary theme of serine protease involvement in situations critical to an animal’s survival, and the efficiency, adaptability and potential to be regulated shown by these enzyme systems, it is proposed that barnacle cement polymerization is driven by a similar enzymatic mechanism to that involved in blood coagulation. A common enzymatic mechanism between these systems may be due to derivation from a single ancestral clotting mechanism with subsequent adaptation to a specific function, in which case highly conserved proteins should exist in both systems. Alternatively, convergent evolution may have occurred whereby a similar enzymatic mechanism arose separately multiple times, and commonality exists due to similar selective pressures on the systems. Systems evolved in such a fashion would have functionally analogous proteins, but the biochemical details of these systems (amino acid sequence, 3D structure, properties) would be distinct.

Amino acid sequence level analyses have shown the proteolytic cascades of vertebrate blood coagulation, horseshoe crab blood coagulation, the complement reaction and drosophila dorsal-ventral patterning to have all evolved from a common ancestral cascade (Krem and Di Cera 2002). Following this, it is hypothesized that proteins involved in both the barnacle cement and blood coagulation systems were derived from a common ancestral mechanism and therefore will share homologous proteins. Evidence is presented here, based on studies utilizing a novel method to obtain unpolymerized barnacle cement (as described in Chapter 2), that polymerization of barnacle cement is biochemically similar to blood coagulation, both in terms of the polymerized protein aggregate and the polymerization mechanism, and that homologous proteins to vertebrate blood clotting factors are found in barnacle cement.

The objectives of this study are targeted at assessing the hypothesis that barnacle cement polymerization and vertebrate blood coagulation occur by a similar enzymatic mechanism and that these systems share homologous proteins. Objectives
are four-fold and include the determination of: 1) if polymerized barnacle cement resembles vertebrate clotted fibrin on an ultrastructural and biochemical level; 2) if anticoagulants with a known mechanism of action are effective at inhibiting cement polymerization; 3) if trypsin activity is present in barnacle cement and if so, if barnacle trypsin is homologous to mammalian pancreatic trypsin; and 4) using tandem mass spectrometry querying the human protein database, if homologous proteins to those involved in vertebrate blood coagulation can be identified in barnacle cement. The conclusions drawn from these four objectives will be used to build a model for barnacle cement polymerization based on evolutionary concepts.
3.2 Materials and Methods

3.2.1 Barnacle Larval Culture, Settlement and Maintenance

The barnacle *Amphibalanus amphitrite* (= *Balanus amphitrite*) (Pitombo 2004) was used for this study. Barnacle larval culture and settlement was conducted at the Duke University Marine Laboratory in Beaufort, North Carolina, following Rittschof et al. (1984a). Barnacle larvae were settled on 7.6 x 15.2 x 0.64 cm glass panels coated with silicone (Dow Corning Silastic T2® or International Veridian®) and maintained in the laboratory as described by Holm et al. (2005).

For experiments conducted at the Naval Research Laboratory (NRL), barnacles on silicone coated panels were transported to the Naval Research Laboratory in Washington, DC after 5 weeks of growth. While at NRL, panels were kept in individual plastic containers filled with artificial seawater (32 ppt, Instant Ocean® in doubly distilled water, aerated overnight before use). Artificial seawater was changed twice a week. At NRL, barnacles were fed with 10 ml dense *Artemia sp.* (Sanders, Morgan, UT, hatched from approximately 1 teaspoon cysts in 1 L seawater) every day for 10 weeks and then every other day thereafter.

3.2.2 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Chemicals were of the highest grade available. Product numbers are provided the first time a chemical is listed.
3.2.3 Unpolymerized Barnacle Cement

For studies utilizing unpolymerized barnacle cement, unpolymerized cement droplets were obtained by hand using a method inspired by Cheung et al. (1977). Cement production is continuous throughout a barnacle’s life (Saroyan et al. 1970), which makes the collection of unpolymerized cement possible. Barnacles were gently removed from a silicone foul-release substrate using a dissecting needle (Hamilton Bell Co. Inc., Montvale, NJ). Barnacles whose base plate broke upon removal were discarded. Immediately following release from silicone, barnacles were classified by cement type (hard or gummy). Barnacles were considered “hard” if there was no opaque, soft cement on the base plate (as determined by visual inspection and gentle probing with a dissecting needle) and were considered “gummy” if >90% of the base plate was covered in opaque, soft cement. Barnacles showing intermediate levels of opaque cement coverage were not used. Only barnacles whose bases were completely in contact with the silicone panel were utilized for this study; barnacles growing in contact with other barnacles or on the edge of the panels were not used. Classification as hard or gummy was conducted immediately upon release since the appearance of soft cement changes as the cement is exposed to air (see Chapter 4).

Following classification, all shell plates (including the base plate) were gently cleaned in deionized water with a cotton swab. Barnacles were then dried with a Kimwipe® and sat in air on a paper towel for 3 hrs. Allowing time for the barnacles to dry is essential for the formation of defined cement droplets. To stimulate release of cement, the periphery of the base plate (where cement is normally released during growth; the junction between the base plate and parietal plates) was gently pricked in an outward direction with a dissecting needle. Opening the cement channels by removing previously polymerized/calcified cement allows for 1-2 μl droplets to form, which can
be taken up with a 0.5 – 10 μl pipettor with a micro tip. Very gently squeezing the barnacle between the thumb and finger (compressing the base plate towards the operculum) increased cement volume. After cement collection, unattached barnacles were maintained in 10.5 cm glass finger bowls for up to two months, with hard and gummy held in separate finger bowls. To prevent strong adhesion to the glass during this time, each barnacle was pushed gently to a different location in its finger bowl daily. Barnacles were used on average once per week for cement collection as described above.

3.2.4 Analysis of Barnacle Cement Using Atomic Force Microscopy

Imaging of barnacle cement was conducted using a Veeco Nanoman Atomic Force Microscope (Digital Instruments, Dimension 3100) in tapping mode. All imaging was conducted in air. Imaging in air is appropriate for hard barnacles due to the fact that: 1) the cement is not highly hydrated; and 2) the cement-surface interface is a dry, solid to solid interface. Imaging in seawater may have led to unnatural changes in cement morphology and possible dissolution of mineralized crystals. Images were extracted using WSxM V. 3.0 Beta 11.1 (Horcas et al. 2007).

Secondary cement and cement droplets were imaged in this study. Secondary cement, used by barnacles for repair and reattachment (Saroyan et al. 1970), was obtained by reattaching barnacles to a glass microscope slide. Barnacles were gently removed from a silicone foul-release substrate using a dissecting needle, dried with a Kimwipe® and placed directly onto a glass microscope slide. Barnacles were allowed to reattach in air for 3 h and immersed in seawater thereafter. After 48 h of reattachment, barnacles were removed from the glass slides using a dissecting needle. Slides were washed lightly with deionized water and the residual cement from five separate barnacles was imaged.
For AFM of cement droplets, four 1 μl cement droplets (collected from hard barnacles) were deposited individually onto 75 x 25 mm glass microscope slides and the droplet was either: 1) immediately covered with a 10 x 30 x 1 mm (l x w x d) glass slide (cut to size with a diamond scribe, placed with approximately 5 mm hanging over the edge of the 75 x 25 mm slide for ease of removal) and placed in seawater to simulate the barnacle-cement-substrate interface; 2) place in seawater uncovered; 3) immediately covered with 10 x 30 x 1 mm glass slide and left to cure in air; or 4) allowed to cure in air, uncovered. After 48 hrs, slides in seawater were removed from seawater, the cover slide was removed from the covered treatments and slides that had been in seawater were washed lightly in deionized water. Each droplet was imaged at 5 different regions within the droplet.

### 3.2.5 Analysis of Barnacle Cement Using Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was performed using Attenuated Total Reflectance (ATR). FTIR was conducted on a Nicolet Magna-IR 750 Spectrometer with a DTGS KBr detector. Germanium ATR crystals (Harrick Model #EJ2122, 45°, 50 x 10 x 2 mm) were used for IR experiments. ATR crystals were reused up to three times, with each crystal cleaned thoroughly with ethanol between assays. The ATR crystal was mounted in a stainless steel Harrick Horizon® multiple reflection ATR accessory, with a water-tight gasket. Prior to placement of barnacles onto the ATR crystal, at least 15 background spectra of the clean ATR crystal were taken over the course of 30 min. All background spectra for each assay were averaged to serve as a baseline for spectral analysis.

To obtain IR spectra of barnacle secondary cement, 5-7 hard barnacles were removed from a T2® silicone coated panel using a dissecting needle, the shell plates of
the barnacles were dried with a Kimwipe® and barnacles were immediately placed on an ATR crystal, on which they would secrete cement. The usable surface area of the ATR crystal measured 50 x 8 mm, which allowed for placement of 5–7 barnacles, depending on barnacle size. Three IR assays with hard barnacles were conducted.

Spectra acquisition began immediately after placement of barnacles on the ATR crystal. Spectra were taken summing either 32 or 16 scans at a resolution of 8 wavenumbers. Spectra were taken every minute for the first 15 min after placement, every 3 min for the next 45 min and every 10 – 15 min for the next 8 hrs. Barnacles were left to reattach in air for up to 23 hrs or until no change was observed in spectra. After this time period, a small amount (1-2 ml) of artificial seawater was added to the trough of the ATR element. Enough seawater was added to cover the barnacle base plates, but not to cover the operculum. A glass microscope slide was placed over the trough of the ATR element to minimize evaporation. Spectra were acquired every 10 – 15 min for 4 hrs while the barnacles’ base plates were covered in seawater.

Of interest to this experiment are spectra of residual cement left by reattaching barnacles. After 4 hrs in seawater, the ATR element was removed from the spectrometer and barnacles were carefully removed from the ATR crystal using forceps. Seawater was poured off the Ge crystal and the crystal was rinsed with a small amount of deionized water and gently blown dry with air. Cement left by reattaching barnacles could clearly be seen on the ATR crystal. The ATR element with residual cement was placed back on the spectrometer. Spectra of residual cement were taken every 15 min for 1 hr or until no changes between spectra were observed.
3.2.6 SDS-PAGE, Native Gel and Western Blotting of Barnacle Cement

3.2.6.1 SDS-PAGE

Barnacle cement proteins polymerize rapidly upon removal from the barnacle base and therefore rapid denaturing of the cement proteins is necessary to prevent polymerization. For SDS-PAGE, reducing sample buffer containing 10% (w/v) SDS and 5% (v/v) β2-mercaptoethanol (Modified from Laemmli 1970) was added directly to unpolymerized cement and samples were heated at 100°C for 4 min. Reducing sample buffer was added in excess (80% total volume rather than 50%) to prevent polymerization of cement proteins. 1-3 μl unpolymerized cement was loaded on each lane. Although the volume of cement varied between gels depending on the application, all lanes within a gel contained the same initial volume of unpolymerized cement. Barnacle cement contains, on average, 10.3 μg protein per microliter, and therefore the protein content in 1 μl cement was sufficient to visualize protein bands with Coomassie Blue stain. Samples were run on a 4-20% gradient gel (Pierce Precise Precast Protein Gel, Product #25224: 12 lane, 30 μl or #25244: 15 lane, 25 μl) along with molecular weight markers (Novagen Trail Mix 10 – 225 kDa Protein Markers, #70980-3) at 40 volts for 15 min and then at 100 volts for 1 hr.

Gels that were not used for Western blotting were stained overnight with Coomassie Blue (0.25% Coomassie Brilliant Blue R-250 (BioRad Electrophoresis grade, #161 0400), 7.5% acetic acid, 5.0% methanol), then destained in a solution of 25% methanol, 7.5% acetic acid for 30 min and finally destained in several changes of 7.5% acetic acid for 24-48 hrs.
3.2.6.2 Native Gel Electrophoresis

For native gel electrophoresis, non-reducing sample buffer (PAGEgel Co. #DB33002) was added to 1.5 μl unpolymerized cement, vortexed, and immediately loaded onto a 3-20% Native gel (PAGEgel Co. Precast Native Gel, #DN32012: 12 well, 35 μl or #DN32027: 17 well, 17 μl). Native gels were run at 100 volts for 15 min, then at 200 volts for 1 hr.

3.2.6.3 Western Blotting

Following SDS-PAGE or native gel electrophoresis, proteins were transferred to a PVDF membrane (Millipore Immobilon P®q, 0.2 μm pore size, #ISEQ 081 00). Proteins were transferred at 4°C at 15 volts overnight. Tris-Glycine transfer buffer (pH 8.3) contained 15% methanol. Gels were stained with Coomassie Blue after blotting to assess protein transfer.

Barnacle cement proteins that had been transferred onto PVDF membranes were blocked to reduce non-specific binding and immunostained with antibodies for fibrinogen or trypsin. Bovine serum albumin (BSA) or non-fat dry milk was used for blocking. TBS buffer (pH 7.6) was used for all immunostaining. Goat polyclonal antibody to full length human fibrinogen (1:8,000 dilution) and rabbit polyclonal antibody to full length bovine pancreatic trypsin (1:10,000 dilution) were used as primary antibodies (Novus Biologicals, Fibrinogen: #NB 600-926, Trypsin: #NB 600-1277). Anti-goat and anti-rabbit polyclonal antibodies (both at 1:20,000 dilution), conjugated to either horseradish peroxidase or alkaline phosphatase were used as secondary antibodies (Novus Biologicals #NB7160, #NB7357, #NB710-AB & #NB730-AB). Antibodies were detected using a TMB or BCIP/NBT substrate (Vector Laboratories, #SK4400 & #SK5400 respectively) depending on the secondary antibody. Endogenous peroxidase activity was not observed on Western blotted proteins. Control
staining for non-specific binding was conducted with secondary antibody only. Positive control proteins (Porcine fibrinogen, fraction I, #F2629; Bovine pancreatic trypsin, TPCK treated, #T1426) were run on SDS-PAGE and native gels and Western blotted along with barnacle cement proteins.

3.2.7 Inhibition of Cement Polymerization by Anticoagulants

Inhibitory affects of anticoagulants on cement polymerization were assayed using three methods: 1) Barnacle reattachment, 2) Optical microscopy, and 3) Electrophoresis of cement with and without anticoagulants.

3.2.7.1 Barnacle Reattachment

The barnacle reattachment assay (Rittschof et al. 2008) allows for rapid assessment of barnacle adhesive strength. In this assay barnacles are grown on a silicone substrate, then removed from the silicone surface using a hand-held mechanical force gauge. Removed barnacles are then placed on another surface to which they are allowed to reattach. As cement production is continuous throughout a barnacle’s life, reattachment is possible and strength of adhesion on silicone after one week of reattachment is the same as adhesion strength of barnacles reared on the silicone substrate. This assay can be used to determine if a coating decreases adhesion strength (in shear) by removing a barnacle from silicone and allowing it to reattach to test and control substrates. Statistical analyses for settlement assays were conducted using SigmaStat® V. 3.10.0 and GraphPad® Prism V. 5.01.

Initial reattachment trials were conducting on six anticoagulants: trypsin inhibitor (soybean, #93620), heparin (sodium salt, porcine intestinal mucosa), EDTA (Fisher Chemical, #BP118), aspirin (Bayer Pharmaceutical), and at a later date warfarin (98%, #A2250) and EGTA (#E4378). Trials with trypsin inhibitor, heparin, EDTA and aspirin...
were tested together along with two controls, deionized water and BSA (protein control, #B4287). For this group of anticoagulants, treatments were coated onto T2 silicone panels (prepared for Holm et al. 2005; 7.6 x 15.2 x 0.64 cm glass panels coated with Dow Corning Silastic T2®). Warfarin and EGTA were tested together at a later date with only deionized water as a control (since neither warfarin nor EGTA are proteins). Due to a concern that molecules released from silicone have an inhibitory effect on cement polymerization (see Chapter 4), warfarin and EGTA treatments with their deionized water control were coated on glass.

Coating of panels with anticoagulant or control treatments was conducted via solution deposition as follows. Each test panel (15 x 7.5 cm) was coated with a single test agent or control by placing up to ten, 0.5 ml droplets of 1 mg ml⁻¹ anticoagulant or control in different locations across the surface of the panel. Warfarin, EDTA and EGTA solutions were stirred for at least 30 min before use. Treatments were allowed to dry thoroughly overnight in a fume hood.

Reattachment of barnacles to test panels was conducted with ~6 mm diameter barnacles, which had been grown on silicone substrates (T2® or Veridian®). Barnacles were removed from silicone using a hand-held mechanical force gauge (Shimpo MF-51b, #93953-10), gently dried with a paper towel and placed onto each panel directly on the dried test solution. Before placement of barnacles on panels, panels were placed individually into 22.5 x 15 x 5 cm (l x w x d) plastic dishes. Barnacles were allowed to reattach in air for 3 hrs, after which time 750 ml seawater was very slowly added to the panels. All barnacles were fed daily over the course of the experiment with 10 ml dense Artemia sp. Following reattachment, removal force was quantified using a hand held mechanical force gauge.

For trypsin inhibitor, heparin, EDTA and aspirin, reattachment trials were conducted for 7 days and then subsequently for 1 day. In order to reduce individual
variation in cement properties among barnacles that may affect adhesive strength, the
same group of barnacles (on the same surface) was used in both 7 and 1 day
reattachment trials. Barnacle bases were lightly sanded with GatorGrit #120 Garnet
sandpaper between trials to remove any calcified cement from the cement channels.
Since adhesive strength was very low for 1 day treatments in the first group of
reattachment assays, warfarin and EGTA reattachment trials were conducted for 5 and
7 days with a separate set of barnacles for each trial. Warfarin, EGTA and their
deionized water control were reattached in low flow running seawater to prevent
potential toxicity of warfarin. Seawater flow was stopped for roughly 1 hr per day
during feeding.

Initial reattachment trials were used to determine which of the anticoagulants
showed the greatest potential to reduce barnacle adhesive strength. Heparin was the
only anticoagulant to show consistently low removal force in both 7 and 1 day
reattachment trials and therefore was selected for further reattachment studies.

Additional reattachment assays with heparin were conducted to assess the
effects of reattachment duration and heparin concentration on barnacle adhesive
strength. The effect of reattachment duration on heparin treated surfaces was assessed
by reattaching the same group of individuals to control and treatment panels for 1, 2, 3
and 4 days (conducted in that order; n = 10 barnacles initially per treatment). The use of
the same barnacles in successive reattachments was intended to reduce individual
variation in cement properties, which may affect adhesive strength. After each
reattachment interval, removal force was quantified, the base was dried gently with a
paper towel and barnacles were immediately placed onto a new panel with the same
treatment for the next reattachment interval. For these reattachment trials, 1 mg ml⁻¹
sucrose (#S579115) was added as a control in addition to deionized water. Sucrose was
used to determine if the presence of a bulk, hydrophilic sugar that is not an
anticoagulant would affect barnacle adhesive strength. The effect of heparin concentration on barnacle adhesive strength was assessed by reattaching barnacles to glass panels coated with heparin at three different concentrations: 0.1, 1 and 10 mg ml\(^{-1}\) (n = 5 barnacles per treatment). Glass panels were used to avoid an inhibitory effect from molecules released from silicone.

### 3.2.7.2 Optical Microscopy

Optical microscopy was used to compare the structure of barnacle cement left on heparin coated glass to barnacle cement left on clean, untreated glass and on BSA coated glass (controls). Heparin was selected for use in this study based on results of reattachment studies showing low removal force of barnacles on heparin coated panels. Barnacles (4 for each treatment) were removed from a silicone substrate and allowed to reattach for 7 days to: 1) a clean glass microscope slide, 2) a glass slide coated with BSA, at 1 and 10 mg ml\(^{-1}\) and 3) a glass slide coated with heparin, at 1 and 10 mg ml\(^{-1}\). Barnacles were removed from their reattaching substrate after 7 days with a dissecting needle and residual cement was imaged on a compound light microscope at 100x magnification.

### 3.2.7.3 Electrophoresis of Cement With and Without Anticoagulants

Barnacle cement polymerizes rapidly in air. As polymerization proceeds, the number and intensity of bands resolvable with SDS-PAGE decreases (see Chapter 4). If anticoagulants inhibit cement polymerization, then the pattern of protein bands after a given incubation time should differ between samples with and without an anticoagulant added.

The effects of anticoagulants on cement polymerization were tested using SDS-PAGE (under reducing conditions) and native gel electrophoresis. Four anticoagulants,
Heparin (sodium salt, porcine intestinal mucosa), trypsin inhibitor (soybean, #93620), warfarin (98%, #A2250), and EDTA (Fisher Chemical, #BP118) were assayed with Native gel electrophoresis. These four anticoagulants in addition to EGTA (#E4378) were assayed with SDS-PAGE. Anticoagulant concentrations were: Heparin, 5 mg ml⁻¹; trypsin inhibitor, 250 ng ml⁻¹; warfarin, EDTA and EGTA, 1 mg ml⁻¹, stirred for at least 30 min before use.

For SDS-PAGE, 1 μl unpolymerized cement was added to 2 μl of each anticoagulant solution. As a control, 1 μl unpolymerized cement was added to 2 μl deionized water. Samples were vortexed, and incubated on ice for 2 min. After 2 min incubation, reducing sample buffer was added, samples were heated and run as described previously. For Native gels, 1 μl unpolymerized cement was added to Native gel sample buffer containing 2 μl anticoagulant or deionized water. Samples were incubated at room temperature for at least 15 min before loading and running as described previously.

Following electrophoresis, gels were stained/destained as described previously. Gels were photographed after destaining with a digital camera. To allow direct comparison of band intensity among lanes, each gel lane was analyzed for pixel intensity using Scion Image® V. Alpha 4.0.3.2 and plotted using SigmaPlot® V. 9.0. SDS-PAGE with anticoagulants was conducted four times and native gel electrophoresis with anticoagulants was conducted twice.
3.2.8 Activity of Trypsin-like Serine Protease(s): Quantification of Trypsin Activity

Trypsin activity can be quantified spectrophotometrically based on tryptic cleavage of arginine esters. BAPNA (Nα-Benzoyl-DL-arginine 4-nitroanilide; Arcos Organics #227740010) was used as a trypsin substrate and prepared at 0.044% (w/v) by first dissolving BAPNA in DMSO (1% v/v) and then adding 50 mM Tris buffer, pH 8.0. Reaction conditions (pH, incubation temperature, buffer concentration) followed Dougherty (1996) who optimized reaction conditions for general protease activity in Chthamalus fragilis unpolymerized cement.

To assay trypsin activity, 6 μl unpolymerized cement was added to 800 μl BAPNA solution. Samples were vortexed and incubated at 37°C for 1 hr. Two controls were run along with cement samples: 1) 6 μl Tris buffer (50 mM, pH 8.0) with 800 μl BAPNA solution to account for background hydrolysis of BAPNA, and 2) 6 μl unpolymerized cement with 800 μl Tris buffer (50 mM, pH 8.0, without BAPNA) to account for any absorbance contribution of unpolymerized cement unrelated to hydrolysis of BAPNA. Control samples were vortexed and incubated at 37°C for 1 hr. Following incubation, all samples were centrifuged at 9000 rpm for 10 min in a Fisher Scientific MicroD centrifuge. Samples were transferred to a quartz semi-micro cuvette (Starna Cells #9-Q-10) and absorbance at 405 nm (referenced to Tris buffer alone) was read on an Hewlett Packard 8451A diode array spectrophotometer. Samples were staggered in ~8 sample groups (each with controls) so that all samples could be read within 10 min of centrifugation.

Calculation of trypsin activity was based on a trypsin standard curve. The standard curve consisted of 1.85 E⁻⁵, 9.81 E⁻⁶, 4.63 E⁻⁶, 2.31 E⁻⁶, 1.16 E⁻⁶, 5.78 E⁻⁷, 2.89 E⁻⁷, and 0 BAPNA units ml⁻¹ trypsin, prepared immediately prior to use with porcine
pancreatic trypsin (Type II-S, #T7409). 6 μl of each standard was combined with 800 μl BAPNA solution, samples were vortexed and incubated at 37°C for 1 hr, centrifuged at 9000 rpm for 10 min, and absorbance was read as described above. Absorbance values for barnacle cement samples were adjusted by subtracting the average absorbance of both controls listed above (BAPNA only and unpolymerized cement in buffer without BAPNA). Using adjusted barnacle cement absorbance values, trypsin activity was extrapolated based on the standard curve. Trypsin activity was calculated for 20 individuals (10 hard and 10 gummy barnacles).

### 3.2.9 Activity of Trypsin-like Serine Protease(s): Barnacle Settlement Assays

Barnacle cyprids settle in response to peptides with carboxyl terminal arginine or lysine residues, which are generated by trypsin-like serine proteases (Tegtmeyer and Rittschof 1988, Pettis 1991). Therefore, settlement in response to small peptides in unpolymerized cement can be used as evidence for the activity of a trypsin-like serine protease in barnacle cement. All statistical analyses for settlement assays were conducted using SigmaStat® V. 3.10.0.

#### 3.2.9.1 Whole Cement Assays

The response of barnacle cyprids to polymerizing cement taken from conspecific adults was tested using a series of settlement assays. For whole cement settlement assays, 1, 3, or 6 μl unpolymerized cement was placed in a 5 cm polystyrene Petri Dish (Falcon #351006) in 1 μl droplets. 5 ml filtered, aged seawater was immediately added to the dish. Approximately 20, newly metamorphosed (day 0) cyprids were added to each dish. Cyprids were allowed to settle for 24 hrs, over which time they were kept at 27-28°C on a 12:12 hour light:dark cycle. Settlement treatments (1, 3 and 6 μl) along
with controls (clean dish, no cement) were conducted in duplicate. The entire assay was conducted three times.

Settlement in all dishes was quantified after 24 hrs. Cyprids that had clearly metamorphosed into a juvenile and cyprids that had cemented to the dish, but had not yet metamorphosed were counted as “settled”. Barnacles were killed by adding a drop of formalyn to each dish, and settled and unsettled barnacles were counted with a dissecting microscope. Barnacle cyprids tend to settle in grooves if no settlement pheromone is present. Therefore location of settlement for each individual was classified as “groove” (where the bottom and side of the Petri dish meet) or “center” (on the flat bottom of the Petri dish).

### 3.2.9.2 Separated Cement Assays

In order to determine which of the cement proteins induce a settlement response, settlement assays were conducted on cement separated by size. SDS-PAGE under reducing conditions (with 2 μl cement per lane) was used to separate proteins by size (conducted as described previously). Proteins were transferred to a PVDF membrane to make cement protein surface available. Settlement response to separated cement was quantified in two ways: 1) settlement on a whole PVDF membrane and 2) settlement on PVDF membranes cut to specific molecular weight ranges.

For settlement on whole PVDF membranes, following protein transfer the membrane was reactivated in methanol and soaked for at least 20 min in several changes of deionized water to remove methanol. An 8 x 5 cm membrane was cut in half and aligned at the bottom of an 8 x 12 x 2 cm (l x w x d) dish comprised of linear polyethylene (the lid from a Fisher brand pipette tip box), so as to have a high and low molecular weight side of the dish. Approximately 200 newly metamorphosed (day 0) cyprids and 100 ml filtered, aged seawater was added to the dish and cyprids were
allowed to settle for 24 hrs at 27-28°C on a 12:12 hour light:dark cycle. After 24 hrs, seawater, unattached cyprids and the membrane were removed from the dish. A 1 cm² grid was drawn onto the dish and the number of settled barnacles in each cm² was quantified. Settlement on whole PVDF membrane was conducted three times.

To increase precision of molecular weight-based settlement response, after protein transfer, PVDF membranes were cut into sections corresponding to specific molecular weight ranges. The PVDF membrane (8 x 10 cm) was placed on top of the stained gel it was run from (separated by plastic wrap) to allow demarcation of lanes. Portions of the membrane that were not in contact with the gel were cut away and discarded. The membrane was then cut vertically every two lanes so as to produce 2 lane wide strips. These strips were then cut horizontally every 1 cm, which yielded 6 approximately 1 x 1 cm squares. After activating in methanol and soaking in several changes of deionized water (for a least 20 min), each 1 x 1 cm PVDF segment was placed in an individual 5 cm Petri dish along with 5 ml filtered, aged seawater and ~20 cyprids. Cyprids were allowed to settle for 24 hrs at 27-28°C on a 12:12 hour light:dark cycle. Settlement was quantified using the same procedure as described for whole cement assays. Four replicate sets of PVDF segments (each with 6, 1 x 1 cm squares ranging from low to high molecular weight) were assayed along with 4 controls (filtered, aged seawater only) using cyprids from the same larval cohort.
3.2.10 Analysis of Cement Proteins Using Tandem Mass Spectrometry

Tandem mass spectrometry was used to determine if direct homologues to human blood coagulation proteins are present in unpolymerized barnacle cement. Mass spectrometry was conducted at the Mass Spec Based Proteomics Facility at the University of Puerto Rico, Rio Piedras Campus. Peptides for analysis were produced through direct trypsin digestion of unseparated cement droplets (in solution) and trypsin digestion of bands isolated from an SDS-PAGE gel (in gel).

In solution trypsin digestion of unpolymerized barnacle cement was conducted by adding 1 μl unpolymerized cement directly to 100 μl 40 mM ammonium bicarbonate with 10% acetonitrile (ACN; mass spectrometry grade). Two, 1 μl cement samples were taken from each of three hard and three gummy individuals (12 samples total). Trypsin Gold (Promega #V5280), reconstituted to 1 μg μl⁻¹ in 50 mM acetic acid, was added immediately to each sample. 2.0 μg trypsin was used for samples from gummy individuals whereas 1.3 μg trypsin was used for samples from hard individuals to account for differences in cement protein quantity between hard and gummy barnacles (see Chapter 4). Samples were incubated at 37°C for 15 hrs, after which time ACN was added to 50% total volume and samples were shipped overnight to the University of Puerto Rico for mass spec analysis as described below.

For in gel digestion, unpolymerized cement from both hard and gummy barnacles was run on SDS-PAGE under reducing conditions (4-20% gradient gel with 1 μl cement per lane) as described previously. Gels were stained overnight with Coomassie Blue R-250 (0.25% Coomassie, 7.5% acetic acid, 5.0% methanol). Gels were shipped via overnight mail to the University of Puerto Rico during staining by placing each gel in a
sealed plastic bag with ~50 ml Coomassie Blue stain. Once in Puerto Rico, gels were
destained in 40% methanol.

Following initial destaining, individual protein bands detected by Coomassie
Blue staining were carefully excised from the gel using a scalpel, destained using 100 mM
ammonium bicarbonate:50% ACN, and then dehydrated in 100% ACN. After removal
of ACN by speed-vacuum, the gel slice was re-hydrated in 40mM ammonium
bicarbonate and 10% ACN. Trypsin (1 μg) was added and incubated overnight (18 h) at
37°C. The tryptic peptides were eluted from the gel slice by incubating the slice in a
solution containing 50% ACN and 5% formic acid for 1 h at room temperature.

The tryptic-peptides from both in gel and in solution samples, were loaded onto
a Surveyor®HPLC system and peptides were eluted, using a gradient of ACN (0%-80%)
in 0.2% formic acid/H₂O, directly into the electro-spray ionization (ESI) source. The
eluted tryptic peptides were infused into a LTQ mass spectrometer (Thermo Fisher) for
tandem mass spectrometry analysis of the proteins of interest.

Tandem mass spectra were extracted by BioWorks® V. 3.2. Charge state
deconvolution and deisotoping were not performed. All MS/MS samples were analyzed
using Sequest® (ThermoFinnigan, San Jose, CA; V. 2.7). Sequest was set to search the
human database (NCBI non-redundant sub-database) assuming the digestion enzyme
trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a
parent ion tolerance of 2.0 Da. Only those identified peptides that pass selection filters
imposed on the database search were taken into consideration for protein identification
(Xcorr higher than 1.5 (+1), 2.0 (+2) or 2.5 (+3); Delta Score >0.1; 10 or more b and y
ions; MS2 intensity of >5x10⁻⁴, peptide probability >E x10⁻²). Additionally, Scaffold®
(V. Scaffold-01_07_00, Proteome Software Inc., Portland, OR) was used to validate
MS/MS based peptide and protein identifications. Peptide identifications were
accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3.2.10.1 Mass Spec Validation: Western Blotting for Factor XIII A1 Subunit

Tandem mass spectrometry identified the presence of Factor XIII (Fibrin stabilizing factor, a transglutaminase), A1 subunit precursor in barnacle cement. Western blotting was used to verify the mass spec results. Cement samples (1 μl from hard barnacles) were denatured in 20 μl reducing sample buffer containing 10% (w/v) SDS and 5% (v/v) β2-Mercaptoethanol and heated at 100ºC for 4 min. After denaturing treatment, samples were sent overnight mail to the University of Puerto Rico for Western blotting.

At the University of Puerto Rico, samples were run on a 10% acrylamide gel at four sample concentrations: the full cement sample (20 μl containing 1 μl cement), 3 μl sample, 2 μl sample and 1 μl sample. A positive control, rabbit Factor XIII A subunit (recombinant protein) was also run. The gel was run at 60 V for 30 min and then 150 V until the dye front was out of the gel. Cement proteins were transferred onto nitrocellulose membrane in tris-glycine transfer buffer containing 16% methanol. Protein transfer was conducted at 300 mA for 2 hrs at 4ºC. Following protein transfer, the nitrocellulose membrane was placed in blocking solution (containing 5% Non fat dry milk) for 1 hr. Rabbit polyclonal antibody to human Factor XIII A subunit (GeneTex Inc., #GTX72947) was used as primary antibody at 1:500 dilution. Goat anti-rabbit HRP-
conjugated antibody (Chemicon) was used as secondary antibody at 1:2000 dilution. Following staining, the membrane was incubated in ECL Plus (GE Healthcare) for 2 min and then exposed to film. Control staining for non-specific binding was conducted with secondary antibody only.

### 3.2.10.2 Verification of Transglutaminase Activity

The presence of Factor XIIIa, a transglutaminase, was shown using mass spectrometry and Western blotting. Transglutaminase activity of unpolymerized cement was verified using a transglutaminase assay kit (Sigma-Aldrich #CS1070). Assays were based on the reaction of transglutaminase with a cadaverine coated 96-well plate. 1 μl unpolymerized cement was used for each test well. Cement was first added to assay buffer in a microfuge tube and centrifuged. Cement in assay buffer was held on ice until all cement samples had been collected, at which time assay buffer containing cement was transferred to plate wells. Blanks (no enzyme present) and positive control (2 milliunits ml⁻¹ transglutaminase from guinea pig liver), each in triplicate, were run along with barnacle cement samples. Since quantification of transglutaminase was based on peroxidase conjugated streptavidin, and endogenous peroxidase activity has been shown for barnacle cement (Chapter 2), OD₄₅₀ values were corrected for barnacle endogenous peroxidase activity. 1 μl of cement in assay buffer from each individual was run in wells both with and without streptavidin-peroxidase added. The mean OD₄₅₀ of blank wells without streptavidin-peroxidase (background absorbance) was subtracted from the OD₄₅₀ of barnacle cement wells without streptavidin-peroxidase to obtain an absorbance value for endogenous peroxidase. The peroxidase correction for each individual was subtracted from the cement well run with streptavidin-peroxidase for that individual to obtain a peroxidase corrected OD₄₅₀ value.
3.3 Results

3.3.1 Structural and Biochemical Similarity Between Polymerized Barnacle Cement and a Fibrin Blood Clot

3.3.1.1 Atomic Force Microscopy: Barnacle Cement Versus a Fibrin Blood Clot

Atomic force microscopy reveals remarkable structural similarity between a vertebrate fibrin blood clot and polymerized barnacle cement (Figure 9). Whole clotted blood is shown in figure 9A. The clot is composed of red blood cells and aggregated platelets, which are held together by a mesh of cross-linked fibrin. Similarly, polymerized barnacle cement is composed of a mesh of cross-linked fibrous protein, which was occasionally shown to bind together crystalline globules as shown in figure 9B. Clotted blood plasma proteins appear as a mesh of cross-linked fibrin (Fig. 9C) closely resembling the fibrous region observed in a polymerized cement droplet (removed from the base of the barnacle and cured in seawater, while covered: Fig. 9D).
Figure 9: Structural similarity between clotted blood and barnacle cement. A) SEM of a fibrin blood clot, showing cross-linked fibrin (middle arrow), red blood cells (bottom arrow) and platelets (top arrow). SEM image courtesy of Electron Microscope Unit, University of Cape Town. B) AFM of barnacle cement left on a glass slide by a hard barnacle that had reattached for 2 days, showing fibrous protein (top arrow) and crystalline globules (bottom arrow). C) Confocal microscope image of a fibrin blood clot prepared by adding Ca^{2+} and thrombin to isolated plasma. Image from Collet et al. 2005, PNAS 102:9133-9137, Copyright (2005) National Academy of Science, USA. D) AFM of a 1 μl droplet of barnacle cement (from a hard barnacle) cured on a glass slide in seawater while covered with a cover slip for 2 days.
3.3.1.2 FTIR: Barnacle Cement Versus a Fibrin Blood Clot

FTIR spectra of clotted fibrin (bovine and porcine) and polymerized barnacle cement are nearly identical in both the number and position of peaks (Figure 10). Within the 8 cm\(^{-1}\) resolution at which barnacle cement spectra were taken, cement peaks are at the same position as fibrin peaks with the exception of the amide I peak (at 1653 for fibrin; 1639 for barnacle cement). The amide I peak for both barnacle cement and fibrin is broad. The intensity of peaks, relative to other peaks in the spectra is very similar between fibrin and barnacle cement.
Figure 10: FTIR of a fibrin blood clot (top) and polymerized barnacle cement (bottom). The amide I, II, & III region is shown (950 – 1800 cm$^{-1}$). Significant peaks are labeled with wavenumbers. FTIR spectra of fibrin are from Bramanti et al. 1997, Biopolymers 41: 545-553, Copyright (1997) Wiley-Blackwell. FTIR spectra of barnacle cement were obtained from residual cement left by reattaching hard barnacles on an ATR crystal. Note that barnacle cement FTIR were taken at 8 cm$^{-1}$ resolution.
3.3.2 Biochemical Similarity to Vertebrate Fibrinogen

Immunoreactivity to full length human fibrinogen antibody was not clear upon chromogenic detection of Western blotted barnacle cement proteins from SDS-PAGE. Native gel electrophoresis was conducted on unpolymerized cement in order to maximize the intensity of fibrinogen immunostaining. Immunoreactivity to fibrinogen antibody was faint, but visible for Western blotted cement proteins from native gel (Figure 11). At >95% peptide probability, tandem mass spectrometry of barnacle cement protein compared to the human database (NCBI non-redundant sub-database) revealed a single matching peptide to fibrinogen \(\alpha\)-chain preprotein and to fibrinogen \(\gamma\)-chain precursor (insufficient for protein identification).

![Figure 11: Immunoreactivity to full length human fibrinogen antibody. Cement protein Western blotted after Native gel electrophoresis (WB) are shown next to a Native gel of unpolymerized barnacle cement (BC), stained with Coomassie Blue.](image)
3.3.3 Inhibition of Cement Polymerization by Anticoagulants

3.3.3.1 Barnacle Reattachment

Six anticoagulants were tested in initial barnacle reattachment trials: trypsin inhibitor, heparin, EDTA, aspirin and separately, warfarin and EGTA (Figures 12 & 13). Heparin was the only anticoagulant to show consistently low removal force as compared to controls for both 7 and 1 day reattachment trials. The same group of barnacles (on the same treatment) was used for 7 and then 1 day trials to reduce individual variation in cement properties, which may affect adhesive strength. Barnacle bases were lightly sanded between trials to reduce influence from previous trials. Reattachment was conducted on T2 silicone for trypsin inhibitor (soybean), heparin, EDTA and aspirin. Kruskal-Wallis one-way ANOVA on ranks did not reveal a significant difference among treatment groups for 7 day reattachment and pre-planned sequential Bonferroni pairwise comparisons (Rice 1989) of individual treatment and control groups were not significant for any comparison at the 0.05 or 0.1 significance level (Figure 12). Only heparin showed a trend toward lower removal force than controls, although this comparison was not significant. Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference among treatment groups for 1 day reattachment ($H = 12.789$, $df = 5$, $p = 0.025$), but pre-planned sequential Bonferroni pairwise comparisons of individual treatment and control groups were not significant for any comparison at the 0.05 or 0.1 significance level (Figure 12). Removal force was notably low for heparin, EDTA and aspirin for barnacles reattached 1 day. For 1 day reattachment, 9 of 10 barnacles reattached on EDTA and heparin treatments showed no adhesive strength upon removal (barnacles were removed from panels without measurable resistance) as compared to only 4 of 10 barnacles showing no adhesive strength for the distilled water control and 6 of 10 on BSA.
Due to a concern that molecules leaching from silicone may inhibit polymerization and effect removal force (see Chapter 4), warfarin and EGTA reattachment trials were conducted on glass. Since adhesive strength was very low for 1 day treatments in the first group of reattachment assays, warfarin and EGTA reattachment trials were conducted for 5 and 7 days with a separate set of barnacles for each trial. Kruskal-Wallis one-way ANOVA on ranks did not reveal a significant difference among treatment groups for 5 or 7 day reattachment ($n = 10 \& 7$ barnacles respectively) and pre-planned sequential Bonferroni pairwise comparisons of individual treatment and control groups were not significant for any comparison at the 0.05 or 0.1 significance level (Figure 13).

Figure 12: Mean removal force (±SE) for barnacles reattached to T2 silicone coated with 1.0 mg ml$^{-1}$ treatment, for 7 or 1 days. Barnacles were first reattached for 7 days, the base was sanded to remove calcified cement from the cement ducts, and the same group of barnacles was immediately reattached for 1 day. dH$_2$O is deionized water. Trypsin inhibitor is from soybean. $n = 10$ barnacles for all treatments.
Figure 13: Mean removal force (± SE) for barnacles reattached to glass coated with 1.0 mg ml⁻¹ treatment, for 5 or 7 days in low flow running seawater. dH₂O is deionized water. n = 10 barnacles for 5 day reattachment and n = 7 barnacles for 7 day reattachment.

Adhesive strength of barnacles reattached to heparin coated panels in initial reattachment trials was quite low for both 7 and 1 day trials, suggesting that further assessment of barnacle adhesive strength on heparin coated panels was warranted. A series of reattachment assays were conducted with heparin to assess the effect of reattachment duration. The same group of barnacles (on the same treatment) was used for each successive reattachment to reduce individual variation in cement properties, which may affect adhesive strength. Barnacles reattached to heparin treatments for 1, 2, 3, or 4 days showed little or no removal force regardless of how long they were reattached (Figure 14). As expected, deionized water controls showed a stepwise increase in removal force with the length of time reattached. Sucrose controls also increased in removal force from 1 to 4 days. A Kruskal-Wallis one-way ANOVA on ranks conducted for 1 day reattachment revealed a significant difference among treatment groups (H = 7.438, df = 2, p = 0.024). Pre-planned sequential Bonferroni pairwise comparisons showed heparin to be significantly different from the sucrose...
control at \( p < 0.05 \). Statistical comparisons were not conducted on 2, 3, and 4 day trials since data may be influenced by previous reattachment trials.

The effect of heparin concentration on barnacle adhesive strength was assessed by reattaching barnacles to glass panels coated with heparin at three different concentrations: 0.1, 1 and 10 mg ml\(^{-1}\). Heparin showed a clear concentration depended decrease in removal force, as shown in Figure 15. Removal force differed significantly among treatment groups (Kruskal Wallis one-way ANOVA on ranks: \( H = 14.508, \text{df} = 3, \ p = 0.002 \)). Pre-planned sequential Bonferroni pairwise comparisons showed the 10 mg ml\(^{-1}\) group to be significantly different than the deionized water control at \( p < 0.05 \).

To calculate the heparin EC\(_{50}\) (the concentration at which removal force is half the maximum removal force on clean glass), a sigmoidal dose-response curve was fitted to the data using GraphPad\textsuperscript{®} Prism V. 5.01. Data was plotted semi-logarithmically (using the log of heparin concentration) with clean glass plotted at 2 log units below the lowest heparin concentration. For 7 day reattachment, heparin EC\(_{50}\) was 0.0237 mg ml\(^{-1}\) (\( r^2 = 0.61 \)).
Figure 14: Mean removal force (±SE) for barnacles reattached to deionized water (dH₂O), sucrose (1 mg ml⁻¹) or heparin (1 mg ml⁻¹) coated T2 silicone over time. The same group of barnacles was successively reattached to the same treatment for 1, 2, 3 and 4 days (in that order). n = 10 barnacles for dH₂O and sucrose, n = 10 for heparin 1 day, n = 9 for heparin 2, 3 and 4 days. Heparin is significantly different from sucrose for 1 day reattachment (pre-planned sequential Bonferroni pairwise comparison: p < 0.05). Statistical comparisons were not conducted on 2, 3, and 4 day trials since data may be influenced by previous reattachment trials.
Figure 15: Mean removal force (±SE) for barnacles reattached to clean glass (marked as 0.00) and glass coated with heparin at three concentrations. Left: Initial removal force from T2 silicone is shown for comparison. * indicates significant difference from the deionized water control group (pre-planned sequential Bonferroni pairwise comparison: p < 0.05).

Right: data fitted with a sigmoidal dose-response curve to calculate EC$_{50}$, $r^2 = 0.61$.

Barnacles were reattached for 7 days. n = 5 barnacles for each concentration.
3.3.3.2 Inhibition of Cement Polymerization by Anticoagulants: Optical Microscopy

Reattachment assays showed weak adhesion of barnacles to heparin coated panels. The effect of heparin on cement structural morphology was assessed using optical microscopy. Residual cement left by barnacles reattached to clean glass, BSA coated glass (1 & 10 mg ml\(^{-1}\) BSA) and heparin coated glass (1 & 10 mg ml\(^{-1}\) heparin) was examined (n = 4 barnacles per treatment). At 100x magnification, residual cement on clean glass and BSA coated glass generally appears as a smooth layer of interlocking fibers (Figure 16A, B & C). The cement layer is most dense at the periphery of the barnacle, where cement is released. Longer fibrous inclusions are visible, but are not the dominant motif. At 100x magnification, residual cement on heparin coated glass shows clear differences in cement structure (Figure 16D, E & F). Cement does not appear as a smooth layer and the distinction between cement released at the periphery of the base and inner regions of the base is less defined than in controls. Long, non-interlocking fibers are abundant, which were observed in one barnacle to occur as a curly ‘hairball-like’ structure (i.e. Figure 16F).

![Figure 16: Residual cement left by barnacles reattaching for one week to: A) clean glass; B) BSA coated glass (1 mg ml\(^{-1}\)); C) BSA coated glass (10 mg ml\(^{-1}\)); D) Heparin coated glass (1 mg ml\(^{-1}\)); E & F) Heparin coated glass (10 mg ml\(^{-1}\)).](image-url)
3.3.3.2 Electrophoresis of Cement With and Without Anticoagulants

For most gels and most anticoagulants, the number and intensity of protein bands resolvable with SDS-PAGE and native gel electrophoresis was greater when cement was incubated in the presence of anticoagulants than when cement was incubated in deionized water. Cement from gummy barnacles showed a greater difference in the number and intensity of resolvable protein bands between anticoagulant treatments and deionized water controls than did cement from hard barnacles.

To allow detailed comparison of band intensity among gel lanes, Coomassie Blue stained SDS-PAGE gels were photographed, each gel lane was analyzed for pixel intensity and the data were plotted. Protein band intensity for a gummy cement SDS-PAGE gel with and without anticoagulants is shown in figure 17. After 2 min incubation time the only visible major protein bands for the deionized water control are at 200 and 30 kDa, whereas in all anticoagulant treatments several other major protein bands, especially in the 150 – 35 kDa range, are visible. Low molecular weight peptides (16 – 10 kDa) are most abundant in the deionized water control. For anticoagulant treatments, protein bands at 200 and 30 kDa are similar in intensity among the anticoagulants. Protein bands in the 150 – 35 kDa range, however, vary in number and intensity and are most numerous and intense for heparin and EDTA.
Figure 17: Protein band intensities for SDS-PAGE gel: cement from gummy barnacles, with and without anticoagulants. 1 μl unpolymerized cement plus either anticoagulant or control (deionized water, dH₂O) was incubated on ice for 2 min before adding reducing sample buffer. Trypsin inhibitor is from soybean. Each peak is a band on the SDS-PAGE gel. After staining with Coomassie Blue, the gel was photographed, each lane was digitally analyzed for pixel intensity and the data plotted.
Protein band intensities for a hard cement SDS-PAGE gel with and without anticoagulants are shown in figures 18 & 19 (shown separately due to a limitation in the number of gel lanes). Similar to SDS-PAGE of gummy cement with inhibitors, the intensity of major protein bands is greater in all anticoagulant treatments than in the deionized water control, especially within the 150 – 35 kDa region. Unlike gummy cement gels, major protein bands are still visible within the 150 – 35 kDa region for the deionized water control after 2 min incubation. Protein bands at 200 and 30 kDa are similar in intensity among the anticoagulants with these protein bands occurring only slightly less intensely in the deionized water control. As in gummy cement SDS-PAGE, protein bands in the 150 – 35 kDa region are most intense for the heparin treatment. The difference in protein band intensity between deionized water and EDTA is not as great as shown for SDS-PAGE of gummy cement. For hard cement, protein bands in the 150 – 75 kDa region are slightly more intense in the EDTA treatment, and several bands in the 75 – 35 kDa region occur in the EDTA treatment that do not occur in the deionized water control.
Figure 18: Protein band intensities for SDS-PAGE gel: cement from hard barnacles, with and without anticoagulants. 1 μl unpolymerized cement plus either anticoagulant or control (deionized water, dH₂O) was incubated on ice for 2 min before adding reducing sample buffer. Trypsin inhibitor is from soybean. Each peak is a band on the SDS-PAGE gel. After staining with Coomassie Blue, the gel was photographed, each lane was digitally analyzed for pixel intensity and the data plotted.
Figure 19: Protein band intensities for SDS-PAGE gel: cement from hard barnacles, with and without anticoagulants. 1 μl unpolymerized cement plus either anticoagulant (EDTA) or control (deionized water, dH₂O) was incubated on ice for 2 min before adding reducing sample buffer. Trypsin inhibitor is from soybean. Each peak is a band on the SDS-PAGE gel. After staining with Coomassie Blue, the gel was photographed, each lane was digitally analyzed for pixel intensity and the data plotted. EDTA is shown separately from other anticoagulants (Figure 18) due to a limitation in the number of gel lanes. Relative intensity of protein peaks (rather than absolute intensity) should be considered since gels differ in staining and photographic conditions.

Native gel electrophoresis of cement proteins after 15 min incubation time showed subtle, but noticeable differences between anticoagulant treatments and deionized water controls (Figure 20). Of note is the region marked “A” in figure 20. Bands are barely visible in this regions for deionized water controls (hard and gummy), but are visible for all anticoagulant treatments. These bands are particularly distinct for hard cement plus heparin and trypsin inhibitor (soybean) and for gummy cement plus warfarin and EDTA.
3.3.4 Presence and Activity of Trypsin-like Serine Protease(s) in Barnacle Cement

Evidence for the presence and activity of trypsin-like serine protease(s) in barnacle cement was produced using: 1) SDS-PAGE, 2) trypsin activity assays, 3) barnacle settlement assays, and 4) Western blotting.

3.3.4.1 SDS-PAGE

Barnacle cement is composed of at least 12 major proteins ranging from 17 – 200 kDa, as shown by SDS-PAGE under reducing conditions (Figure 21). SDS-PAGE of cement denatured with reducing sample buffer and heat, quickly after removal from the barnacle base shows multiple doublets and an abundance of small peptides (visible as a cloud at the bottom of the gel) upon Coomassie Blue staining.
Figure 21: SDS-PAGE (reducing conditions) of unpolymerized barnacle cement (BC) and molecular weight markers (MW), labeled in kDa. 1 μl unpolymerized cement from a hard barnacle. Reducing sample buffer was added quickly to unpolymerized cement after removal from the barnacle base, and the sample was heated at 100°C for 4 min. Gel was stained with Coomassie Blue.
3.3.4.2 Quantification of Trypsin Activity

Trypsin activity was shown in unpolymerized barnacle cement samples using BAPNA, an arginine ester substrate. Average trypsin activity for unpolymerized barnacle cement was \(1.64 \times 10^{-6}\) BAPNA units \(\mu\text{l}^{-1}\text{cement}\) (SEM \(\pm 1.83 \times 10^{-7}\)), when incubated for 1 hr at 37°C, pH 8.0 (\(n = 20\) barnacles, 10 hard, 10 gummy). Trypsin activity ranged from \(7.69 \times 10^{-7} - 4.25 \times 10^{-6}\) units \(\mu\text{l}^{-1}\text{cement}\). Absorbance values for unpolymerized cement with BAPNA were adjusted to account for autohydrolysis of BAPNA substrate and any contribution to absorbance at 405 nm from the cement alone (which was very minor). Note, 1 BAPNA unit is approximately 9000 BAEE units.

3.3.4.3 Barnacle Settlement: Whole Cement Assays

The presence of cement droplets in assay dishes caused a dramatic increase in cyprid settlement at all cement volumes tested (Figure 22). Total percent settlement differed significantly among 1, 3, 6 \(\mu\text{l}\) cement and control groups (Kruskal-Wallis one-way ANOVA on ranks: \(H = 11.47; \text{df} = 3; p = 0.009\)). All treatment groups (1, 3, and 6 \(\mu\text{l}\)) showed a greater total percent settlement than did the control group (SNK pairwise comparison: \(p < 0.05\)).
Figure 22: Total percent cyprid settlement. Settlement assays were conducted for 24 hrs with newly metamorphosed cyprids. Mean and standard error are shown. Groups marked with different letters are significantly different as shown by SNK pairwise comparison. n = 5 assays per cement volume.

In addition to increasing total settlement, the presence of cement droplets caused a shift from settlement only in grooves (where the bottom and side of the Petri dish meet) to settlement on the flat bottom of the Petri dish as well as the grooves of assay dishes (Figure 23). Barnacle cyprids tend to settle in grooves if no settlement pheromone is present. The percent settlement in grooves differed significantly among treatment and control groups (Kruskal-Wallis one-way ANOVA on ranks: $H = 12.32; df = 3; p = 0.006$). 100% of settlement in controls occurred in grooves. All treatment groups (1, 3,
and 6 μl) showed lower percent settlement in grooves than the control group (SNK pairwise comparison: p < 0.05).

Figure 23: Percent cyprid settlement in grooves of the assay dish. Settlement assays were conducted for 24 hrs with newly metamorphosed cyprids. Mean and standard error are shown. Groups marked with different letters are significantly different as shown by SNK pairwise comparison. n = 5 assays per cement volume.
3.3.4.4 Barnacle Settlement: Separated Cement Assays

Barnacle settlement assays with unpolymerized cement, separated by molecular size on SDS-PAGE and transferred to PVDF membranes, revealed a settlement stimulation by low molecular weight proteins. In all assays, very few cyprids settled on the PVDF membrane.

In whole PVDF membrane assays, cyprids settled in high numbers on the flat bottom and upper sides of the low molecular weight end of the 8 x 12 cm assay dish (Figure 24A & B, shown for the trial with the highest total settlement). A chi-square test was used to compare the observed frequency of settlement per cm² of the dish to the expected settlement frequency if settlement had occurred randomly (based on the Poisson distribution). For two of the three whole PVDF membrane assays conducted, the observed settlement frequency differed significantly from expected ($\chi^2$: $p < 0.001$; Figure 24C) and settlement was by far the greatest on the portion of the dish containing the low molecular end of the PVDF membrane. Dividing the dish into thirds (high molecular weight, middle and low molecular weight) and adding settlement per cm² in each third, showed 81% and 78% of total settlement occurring on the low molecular weight third of the dish for these two assays. On the third whole PVDF membrane settlement assay overall settlement was quite low. For this assay the observed settlement frequency again differed significantly from expected ($\chi^2$: $p < 0.01$), but the preference towards the low molecular weight end of the dish was not clear (37% in the low molecular weight third, 22% in the middle third and 41% of total settlement in the high molecular weight third of the dish).
Figure 24: Cyprid settlement on cement separated by size and transferred onto a PVDF membrane. A) Settled barnacles on the high molecular weight end of the dish. B) Settled barnacles on the low molecular weight end of the dish. C) Observed versus expected settlement frequency, based on a Poisson distribution. Observed settlement differed significantly from expected ($\chi^2$: p < 0.001).
To increase precision of the separated cement settlement response, after barnacle cement protein transfer, PVDF membranes were cut into 1 x 1 cm segments corresponding to specific molecular weight ranges (> 125, 125-55, 54-29, 28-16, 15-5 and < 5 kDa). The proportion of cyprids settled was greatest in low molecular weight (15 – 5 kDa) segments, followed by 28 – 16 kDa segments (Figure 25). Settlement on molecular weight range segments was conducted for 4 replicate segments with larvae from the same larval cohort. For each molecular weight range, settled and unsettled cyprids were summed among replicates and statistical analysis was carried out using a contingency table analysis (Chi-square, Fisher’s Exact test) with a Tukey-type pairwise comparison (Zar 1999). The proportion of cyprids settled differed significantly among the six molecular weight ranges and the seawater only control ($\chi^2 = 34.2$, df = 6, p < 0.001; Figure 25). The proportion of cyprids settled in the 15 – 5 kDa range differed significantly from all molecular weight segments >29 kDa and the seawater only control (Tukey-type pairwise comparison: p < 0.05).
Figure 25: Cyprid settlement on segments of PVDF membrane with transferred barnacle cement proteins, corresponding to specific molecular weight ranges. Settlement assays were conducted for 24 hrs with newly metamorphosed cyprids. Proportion of cyprids settled is summed over four replicates of each molecular weight range and control (seawater only). Groups marked with different letters are significantly different as shown by Tukey-type pairwise comparison (p < 0.05).
3.3.4.5 Immunostaining: Bovine Pancreatic Trypsin Antibody

Immunoreactivity to full length bovine pancreatic trypsin antibody was shown at 90 kDa (Figure 26). Staining was visible upon chromogenic detection with TMB substrate. Staining occurred either as a single band at 90 kDa, or as a doublet at 90 and 87 kDa. Staining was observed for unpolymerized cement from both hard and gummy barnacles. Secondary antibody only controls did not stain.

![Figure 26: Immunoreactivity to trypsin antibody. Western blotted cement proteins (WB) are shown next to SDS-PAGE (reducing conditions) of cement proteins (BC) and molecular weight markers (MW), labeled in kDa.](image)
3.3.5 Presence and Activity of Factor XIII (Fibrin Stabilizing Factor, Transglutaminase) in Barnacle Cement

Tandem mass spectrometry was used to determine if direct homologues to human blood coagulation proteins are present in unpolymerized barnacle cement. MS-MS spectra were compared to the human database (NCBI non-redundant sub-database). Proteins identified at greater than 99.0% probability and with two or more matching peptides were accepted. Of interest to this study, coagulation factor XIII A1 subunit precursor (accession number NP_000120.1) was identified with two matching peptides (Figure 27A & B). Factor XIII A is the active subunit of the final enzyme in the blood coagulation cascade. It is a transglutaminase that covalently cross-links fibrin monomers. The presence of coagulation factor XIII A1 subunit in barnacle cement was confirmed with Western blotting using anti-human factor XIII A subunit antibody (Figure 27C).

Transglutaminase activity in unpolymerized cement was verified using a transglutaminase activity kit (Figure 28). Activity was shown for all individuals assayed (8 hard, 11 gummy). Mean OD_{450} for unpolymerized cement from all individuals was 0.282 (SEM ± 0.021) as compared to blanks at 0.1271 (SEM ± 0.004) and positive control (2 milliunits ml⁻¹ transglutaminase) at 0.449 (SEM ± 0.053).
Figure 27: Identification of human coagulation factor XIII (fibrin stabilizing factor), A1 subunit precursor homologue in barnacle cement. A) Identified peptides by tandem mass spectrometry. Identification was made by comparing the MS-MS spectra to the human database. The accession number for the identified protein is NP_000120.1 and corresponds to the human coagulation factor XIII A1 subunit precursor. B) The corresponding MS (inset) and MS-MS spectra for the peptide in bold is shown. Arrow indicates the precursor mass in the MS spectra. The b and y ions obtained upon fragmentation of the precursor ion by collision induced dissociation are shown. C) The presence of the human coagulation factor XIII A subunit homologue in barnacle cement was validated by Western blot analysis using anti-human factor XIII A subunit antibody.
Figure 28: Mean transglutaminase activity (± SE) for unpolymerized cement from 11 gummy and 8 hard barnacles. Assay was conducted using a cadaverine coated 96-well plate. Mean and SEM for blank (no enzyme present) and positive control are shown for comparison.
3.4 Discussion

Polymerization of barnacle cement bears a striking biochemical resemblance to vertebrate blood coagulation, in terms of both the polymerized protein aggregate and the polymerization mechanism. Atomic force microscopy and Fourier transform infrared spectroscopy revealed pronounced structural similarities between polymerized barnacle cement and a fibrin blood clot, with both occurring as an aggregate of fibrous protein. Inhibitors of vertebrate blood coagulation impeded the polymerization of barnacle cement, with heparin (which activates serine protease inhibitor antithrombin III in vertebrate blood coagulation) inhibiting cement polymerization to the greatest extent. Trypsin-like serine protease(s), the primary driver of blood coagulation, were present and active in barnacle cement using SDS-PAGE, enzymatic activity assays, and cyprid settlement assays. A 90 kDa trypsin-like protein was shown to be homologous to bovine pancreatic trypsin with Western blotting. Trypsin activity is likely to activate cement structural precursors, allowing for loose assembly with other structural proteins and rearrangement with the surface. A homologous protein to the catalytic subunit of human coagulation factor XIII (fibrin stabilizing factor: a transglutaminase that catalyzes covalent cross-linking between fibrin monomers) was shown to be present and/or active in barnacle cement using tandem mass spectrometry, Western blotting and transglutaminase activity assays.

3.4.1 Polymerized Barnacle Cement: Structural and Biochemical Similarity to a Vertebrate Fibrin Clot

The ultrastructure of a vertebrate blood clot is remarkably similar to that of polymerized barnacle cement. A vertebrate blood clot is composed of red blood cells and aggregated platelets, which are held together by a mesh of cross-linked fibrin (e.g. Mardel et al. 1998, Kawasaki et al. 2004). Similarly, polymerized barnacle cement is
composed of a mesh of cross-linked fibrous protein, which can bind together crystalline globules, likely calcium carbonate (rhombahedral crystals in figure 7A are morphologically similar to those shown for CaCO3 by Gabrielli et al. 2003). In both cases, a mesh of fibrous protein is interacting with non-soluble components of the system. Clotted blood plasma (liquid portion of blood) appears as a mesh of cross-linked fibrin monomers (Collet et al. 2005) closely resembling the fibrous region observed for a cement droplet polymerized in seawater, while covered.

AFM observations showing that barnacle cement is composed of a tightly interlocking network of fibrous protein are in accord with Wiegemann and Watermann (2003) and Liedert and Kesel (2005) who showed a mesh of interlocking adhesive fibers using SEM and AFM on the base plate and residual cement of barnacles grown on PDMS (polydimethylsiloxane). Based on SEM and AFM, Wiegemann and Watermann (2003) note that the cement fibers may be composed of smaller (20 – 50 nm) globules. The concept that barnacle cement fibers are composed of smaller globular proteins is consistent with the current model for fibrin monomer assembly where a “trinodal” fibrinogen molecule, consisting of three globular domains, is activated by thrombin to allow for electrostatic interactions between neighboring domains (Doolittle 1984, Weisel 1986, Weisel et al. 1999, Sit and Marchant 2001). Interactions between neighboring domains result in a “half-staggered” strand structure (Weisel 1986), which leads to a “bead on a string” type structure (Sit and Marchant 1999, 2001).

In addition to AFM, IR spectroscopy was used to assess structural similarity between polymerized barnacle cement and clotted fibrin. IR spectroscopy can provide detailed information on molecular conformation and chemical bonding of surfaces and interfaces. The FTIR spectra of polymerized barnacle cement is nearly identical to that of clotted bovine and porcine fibrin, as reported by Bramanti et al. (1997). Within the 8 cm⁻¹ resolution at which barnacle cement spectra were taken, cement peaks are at the
same position as fibrin peaks with the exception of the amide I peak (at 1653 for fibrin; 1639 for barnacle cement). The intensity of peaks, relative to other peaks in the spectra is very similar between fibrin and barnacle cement. Although IR cannot be used to unambiguously identify a protein, especially when a mix of proteins is present (as is the case for barnacle cement), IR can provide information on the secondary structure of the protein present (Jackson and Mantsch 1995, Barth and Zscherp 2002). Similar to clotted fibrin (Bramanti et al. 1997) the amide I peak of polymerized barnacle cement is quite broad, indicating that a mix of secondary structures (α-helix, β-sheet, β-turns, random coil) are present in the cement as opposed to a single dominant secondary structure.

Biochemical similarity between barnacle cement proteins and human fibrinogen was assessed using immunostaining and tandem mass spectrometry. Although the intensity of staining was low, staining was observed for cement proteins separated by native gel electrophoresis. At the 95% peptide probability level, tandem mass spectrometry revealed only one matching peptide to both the α and γ-chains of fibrinogen, which is not sufficient to identify the presence of a protein. Despite the structural similarity observed between polymerized barnacle cement and clotted fibrin, the amino acid sequence of the fibrous barnacle cement protein is likely distinct from vertebrate fibrinogen. The clottable protein in barnacle cement should more closely resemble crustacean clottable proteins, such as those in the lobster _Panulirus interruptus_ (Fuller and Doolittle 1971a), the crayfish _Pacifastacus leniusculus_ (Kopacek et al. 1993, Hall et al. 1999) and the sand crab _Ovalipes bipustulatus_ (Madaras et al. 1981).

Biochemical analyses have shown the crustacean clottable proteins (in some texts referred to as fibrinogen) to be homologous among crustaceans (Kopacek et al. 1993, Hall et al. 1999) but biochemically distinctive from vertebrate fibrinogen (Fuller and Doolittle 1971a, Ravindranath 1980, Doolittle 1987). Vertebrate fibrinogen-like protein domains have indeed been identified in invertebrates (echinoderms, insects and
molluscs) but are involved in innate immunity rather than clotting (Adema et al. 1997, Krem and Di Cera 2002).

### 3.4.2 Inhibition of Cement Polymerization by Anticoagulants

Medical research on blood coagulation has led to the identification of a large number of anticoagulants. The mechanism of action for each of these anticoagulants has been well studied and each anticoagulant targets the blood coagulation system in a different way. Common drugs used as anticoagulants include glycosaminoglycans (heparin sulfate, dextran sulfate), coumarin drugs (Dicoumarol, Warfarin), metal chelators (EDTA, EGTA, citrate) and platelet inhibitors (aspirin), among others. Heparin, which is produced naturally by the body, functions by activating antithrombin III (reviewed in Capila and Linhardt 2002). Antithrombin III is a serine protease inhibitor that prevents the activity of thrombin and factor Xa, thereby preventing the formation of a fibrin clot. Heparin also binds Ca^{2+} ions (Nieduszynski 1989, Landt et al. 1994, Rabenstein et al. 1995, Karpukhin et al. 2006), which are essential to serine protease and transglutaminase activity. For thrombin and factors VII, IX, and X, γ-carboxylation of glutamic acid residues during synthesis is necessary for Ca^{2+} binding. Coumarin drugs inhibit the recycling of vitamin K, an essential cofactor to γ-carboxylation (Ansell et al. 2004). Metal chelators bind Ca^{2+}, which limits availability of Ca^{2+} to enzymes. Binding of Ca^{2+} to blood coagulation factors VII, IX, XI, X, XIII and thrombin is essential to their active conformation (Davie and Fujikawa 1975). Lastly, aspirin inhibits the activation of platelets, which prevents the formation of a platelet plug (a precursor to a vertebrate fibrin clot, Szczechlik et al. 1992).

If the mechanism of barnacle cement polymerization is biochemically similar to that of vertebrate blood coagulation as hypothesized, then anticoagulants that disrupt the coagulation of blood should likewise disrupt the polymerization of barnacle cement.
A summary of anticoagulant assay results along with anticoagulant mechanisms of action are shown in Table 2. Anticoagulants do indeed inhibit cement polymerization, suggesting the involvement of: 1) trypsin activity (based on heparin and trypsin inhibitor activity) and 2) Ca\(^{2+}\) as a protease and/or transglutaminase cofactor (based on heparin, EDTA and EGTA activity). The importance of Ca\(^{2+}\) to barnacle cement proteolytic activity (Dougherty 1996, 1997), and to barnacle blood coagulation (Fitzgerald 1968) has been shown previously. Vitamin K may also be a cofactor (based on warfarin activity in some assays), although the activity of warfarin is expected to be less effective than other anticoagulants in these tests, as it inhibits proper synthesis of coagulation factors (through prevention of vitamin K recycling) rather than direct inhibition (Ansell et al. 2004).

Table 2: Summary of Anticoagulant Assays. Success in SDS-PAGE is defined as protein bands in the 150 – 35 kDa region appearing at an intensity greater in anticoagulant treatment than in control.

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Mechanism</th>
<th>Reattachment</th>
<th>SDS-PAGE</th>
<th>Native Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>Inhibitor Activation/Ca(^{2+}) binding</td>
<td>Yes</td>
<td>Most Assays</td>
<td>Yes</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>Inhibit Trypsin Activity</td>
<td>No</td>
<td>Most Assays</td>
<td>Yes</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Prevent Platelet Aggregation</td>
<td>Low in 1 day</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Warfarin</td>
<td>Inhibit Vitamin K Utilization</td>
<td>Unclear</td>
<td>Half of Assays</td>
<td>Yes</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ca(^{2+}) Binding</td>
<td>Low in 1 day</td>
<td>Half of Assays</td>
<td>Yes</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ca(^{2+}) Binding</td>
<td>Unclear</td>
<td>Most Assays</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The inhibition of cement polymerization was more easily detectable at the biochemical level with gel electrophoresis than at the mechano-physical level in
reattachment assays. While all anticoagulants showed some degree of inhibitory activity in SDS-PAGE and native gel electrophoresis, removal force (as shown by reattachment assays) was only consistently reduced in heparin treatments. This difference is likely due to the time scale of polymerization, the ratio of inhibitor to cement, and the precision and sensitivity of the methods. When electrophoresis of cement with and without anticoagulants was conducted, the volume of cement and inhibitor was fixed and short term changes in polymerization were tracked (2 – 15 min). Reattachment assays tracked changes over the course of 1-7 days, over which time the ratio of inhibitor to cement likely decreased, since the volume of inhibitor is fixed (the amount coated on the panel) but the volume of cement will increase as the barnacle re-releases cement. A clear trend of increasing removal force with increasing number of days reattached for barnacles on control panels provides evidence that the barnacle successively releases new cement over the course of days. Hence new cement may not have come into contact with inhibitor. This may explain why certain inhibitors (EDTA and aspirin) showed low removal force as compared to controls for 1 day treatments but not for 7 day treatments.

Of the anticoagulants tested, heparin showed the most extensive and consistent inhibitory effect on cement polymerization. Barnacle cement is a multicomponent system (Kamino 2006) as is its polymerization (as shown in this document). Therefore a broadly active inhibitor, such as heparin (Capila and Linhardt 2002) will show greater inhibition of cement polymerization than inhibitors that only target a specific component of the system. Heparin’s primary mechanism of action in the blood coagulation cascade is through the binding of antithrombin III, causing accelerated formation of an inactive complex with thrombin and most other coagulation factors (Rosenberg and Damus 1973, Capila and Linhardt 2002). In addition, heparin has the capability to bind directly to thrombin (Pochon et al. 1982, Lambin et al. 1984), and is also known to bind Ca^{2+}

In the barnacle cement polymerization system, heparin has the potential to activate serine protease inhibitors (which are likely to be present in the system to regulate trypsin-like serine protease activity), bind directly with proteases and cement components and bind Ca\(^{2+}\), thereby reducing the activity of Ca\(^{2+}\) dependent enzymes (trypsin-like proteases and transglutaminase). It is suggested that trypsin activity in barnacle cement serves a similar biochemical role in cement polymerization as it does in blood coagulation, i.e. activation of structural precursors. Reducing trypsin-like enzyme activity will decrease the number of activated cement precursors and therefore limit the ability of cement proteins to assemble with other structural protein and rearrange with the surface, resulting in decreased adhesion and altered cement structure (as shown by optical microscopy). In reattachment assays, heparin reduced removal force in a concentration dependent manner indicating that successive addition of inhibitor will lead to a corresponding decrease in the amount of activated cement precursors that are available for rearrangement with the surface and cross-linking. Time course assays with barnacles reattaching to heparin coated surfaces for 1, 2, 3, and 4 days showed no increase in removal force with increasing time reattached. Heparin is either still available on the surface for interaction with new cement released by the barnacle, or the adhesion derived from new cement over the course of 4 days is insufficient to counteract the lack of adhesion in cement initially released by the barnacle upon reattachment and in contact with heparin.
3.4.3 Presence and Activity of Trypsin-like Serine Protease(s) in Barnacle Cement

Trypsin-like serine proteases are the primary driver of vertebrate blood coagulation. Therefore, identifying the presence and activity of trypsin-like serine protease(s) in barnacle cement is central to assessing the hypothesis that barnacle cement polymerization and vertebrate blood coagulation occur by a similar enzymatic mechanism and that these systems share homologous proteins. Four independent methods were utilized to assess trypsin presence and/or activity: SDS-PAGE, enzymatic activity assays, cyprid settlement assays and Western blotting, with each method lending support to the hypothesis that trypsin-like enzyme(s) are present and active in barnacle cement and Western blotting supporting homology to bovine pancreatic trypsin.

SDS-PAGE of unpolymerized barnacle cement reveals multiple doublet bands and an abundance of small peptides, visible as a cloud at the bottom of the gel. This pattern is characteristic of protease clipping and activation of proteins, with the larger band as the inactive form and the smaller band the active form of the enzyme (Ojha 1996, Biro et al. 2003). Small peptides resulting from proteolytic cleavage migrate to the bottom of the gel. Generation of activated proteases and peptide clips will occur between the time cement is released by the barnacle and when the cement is denatured for SDS-PAGE. The pattern of SDS-PAGE banding observed is consistent with what has been shown previously for barnacle cement (Naldrett 1993, Kamino et al. 1996, Naldrett and Kaplan 1997, Kamino et al. 2000, Kamino 2001, 2006). Since these studies utilized polymerized cement rather than unpolymerized cement as was used in this study, fewer bands were observed and the presence of doublets was not as clear. As was shown in electrophoretic assays (and will be shown in polymerization assays: Chapter 4), cement proteins polymerize within minutes to the point that they cannot be
rendered soluble in denaturing treatments, making the presence of doublet bands (especially in the 150 – 35 kDa region) less apparent. The number of protein bands resolved with SDS-PAGE is comparable to that described by Dougherty (1996, 1997; up to 22 bands) for unpolymerized cement from *Chthamalus fragilis*.

Largely due to the difficulties associated with acquiring unpolymerized cement, only two previous studies have focused on the enzymes involved in barnacle cement polymerization. Dougherty (1996, 1997) considered protease activity in unpolymerized cement of the barnacle *Chthamalus fragilis* using a FTC-casein substrate. Protease activity was shown in *C. fragilis* cement and activity was enhanced in the presence of Ca$^{2+}$ ions. Using protease inhibitors and dye-labeled PepTag peptides, Dougherty (1996, 1997) showed the activity of a zinc metalloprotease with a preference for carboxyl-terminal basic amino acids. Dougherty (1996) notes that serine protease inhibitors (PMSF and leupeptin) did not inhibit enzyme activity, suggesting that the enzyme present was not a serine protease. In the current study, trypsin-like activity was shown in *A. amphitrite* cement by reactivity towards BAPNA (arginine ester), inhibition by trypsin inhibitor and cyprid settlement assays, which detect serine protease generated peptides (reviewed in Rittschof and Cohen 2004).

Proteases that cut at the carboxyl-terminus of basic amino acids (trypsin-like serine proteases) result in peptides that are signal molecules in a variety of systems (reviewed in Rittschof 1990, 1993, Rittschof and Cohen 2004). These systems include prey location by gastropods, hermit crab shell location, larval release in decapod crustaceans, mammalian complement cascade, and induction of larval settlement in barnacles. Synthetic analogues of mammalian C3a and C5a arginine terminal peptides in the form of basic-basic and neutral-basic dipeptides and neutral-neutral-basic tripeptides were shown to induce barnacle settlement (Tegtmeyer and Rittschof 1988, Pettis 1991). Removal of the carboxy-terminal amino acid with carboxypeptidase A
destroys biological activity (Rittschof et al. 1984b). The importance of a basic amino acid at the carboxy-terminus, as well the ability to produce signal molecules by addition of exogenous trypsin (Rittschof et al. 1990; Ziegler et al. Submitted), suggests that these peptides are generated by trypsin-like proteases.

In this study, barnacle cyprid settlement in response to small peptides released from polymerizing cement was used as evidence for the activity of a trypsin-like serine protease. Cyprids settling in the presence of polymerizing cement showed a clear pheromone induced settlement response, with cyprids setting in the center rather than the edges of the assay dish and settling at a proportion 3.5 - 4.5 times higher than that of controls. Specificity towards low molecular weight peptides was shown using settlement assays on cement proteins separated by weight and transferred onto PVDF membranes. It is interesting to suggest that the process of cement polymerization subsequently induces cyprid settlement by the release of peptide pheromones and therefore could play a key role in structuring intertidal communities. The barnacle surface bound settlement cue (SIPC: settlement inducing protein complex) has recently been characterized and shows significant sequence homology to \( \alpha_2 \)-macroglobulin (Dreanno et al. 2006b). Dreanno et al. (2006a, 2006b) suggest that proteolytic processing of the SIPC by bacteria may produce waterborne settlement pheromones. As shown in Chapter 2, SIPC was identified by tandem mass spectrometry in barnacle cement. It is likely that endogenous trypsin activity involved in cement polymerization, rather than bacterial proteolytic activity, on the SIPC results in the release of waterborne settlement cues.

The presence of an \( \alpha_2 \)-macroglobulin-like protein (A2M) in barnacle cement is particularly noteworthy in regards to the hypothesis that barnacle cement polymerization is biochemically similar to vertebrate blood coagulation. A2Ms function as protease inhibitors to a variety of proteases by steric shielding (Sottrup-Jensen 1989),
are involved in innate immunity (Armstrong and Quigley 1999) and are related to complement cascade proteins C3, C4 and C5 (Sottrup-Jensen 1987). A2Ms are thought to be involved in the regulation of coagulation and fibrinolytic cascades through inhibition of thrombin, factor Xa and plasmin (Steinbuch et al. 1967, Meijers et al. 1987, De Boer et al. 1993). The A2M (SIPC) in barnacle cement may play a role in regulation of cement proteolytic activity through steric inhibition, thereby altering the rate of cement polymerization. Dreanno et al. (2006b) note that the internal thioester group, which allows for covalent binding of protease substrates, is not present in barnacle A2M. The “bait region”, responsible for trapping of proteolytic substrates however, is present and shares significant structural similarity to A2M of the horseshoe crab. As shown for chicken egg macroglobulin, A2Ms lacking the internal thioester group can retain the ability to bind proteases through non-covalent entrapment (Kitamoto et al. 1982, Nagase and Harris 1983, Nagase et al. 1983, Nielsen and Sottrup-Jensen 1993).

A trypsin-like protein occurring as a single band at 90 kDa or a doublet at 90 and 87 kDa was identified in unpolymerized barnacle cement using Western blotting. This protein appears within the region that shows the greatest changes in solubility upon cement polymerization (150 – 35 kDa). Remarkably, immunoreactivity was shown towards full length bovine trypsin immunogen. The trypsin-like serine proteases comprising the blood coagulation cascade in vertebrates are all derived from pancreatic trypsin (Neurath 1984, 1986) and range in molecular weight from 50 – 160 kDa (Davie and Fujikawa 1975). The specificity of coagulation factors is derived from large amino-terminal extensions (nonproteolytic domains) added onto the catalytic region (Patthy 1993, Neurath 1999). Amino-terminal extensions vary greatly between factors and mediate interactions with substrates, cofactors and inhibitors. The molecular architecture of the barnacle cement trypsin-like protein is homologous to the blood coagulation factors in terms of the catalytic region, as shown by immunoreactivity to
bovine pancreatic trypsin. Further characterization of the barnacle cement trypsin-like protein using immunologic assays to specific blood coagulation factors will shed light on the structure of the amino-terminal extension and therefore the potential for specific interactions with cofactors, substrates and inhibitors.

3.4.4 Presence of Factor XIII (Fibrin Stabilizing Factor) in Barnacle Cement

Tandem mass spectrometry was used to further assess the hypothesis that barnacle cement polymerization and vertebrate blood coagulation occur by a similar enzymatic mechanism. Specifically, this tool was used to determine if homologous proteins to human blood coagulation factors are present in unpolymerized barnacle cement. Remarkably, human Factor XIII A1 subunit precursor (the catalytic subunit of ‘fibrin stabilizing factor’, a transglutaminase) was identified and its presence was externally verified with Western blotting. This protein was found at nearly the same molecular weight in barnacle cement as in human plasma (75 kDa in barnacle cement versus 83 kDa in human plasma), and transglutaminase activity was verified with activity assays.

Factor XIII plays a critical role in vertebrate blood coagulation by stabilizing the fibrin clot (Lorand et al. 1962, Laki 1972, Mann 1993). The coagulation cascade culminates in the conversion of fibrinogen to fibrin by thrombin (Davie and Rantoff 1964, Rantoff 1972), allowing for loose assembly of neighboring fibrin monomers via electrostatic interactions (Weisel 1986, Pratt et al. 1997, Yee et al. 1997). Factor XIII, which is normally present in blood plasma in an inactive form, is proteolytically activated by thrombin in the presence of Ca$^{2+}$ (Lorand and Konishi 1964). Activated XIII serves to catalyze the covalent cross-linking of fibrin monomers thereby reinforcing the integrity of the clot and increasing resistance to lysis (Lorand et al. 1962, Mann 1993).
Factor XIII acts as a transglutaminase, creating a \( \varepsilon-(\gamma\text{-glutamyl})\text{lysine} \) cross-link between neighboring glutamine and lysine residues (Pisano et al. 1968).

Covalent cross-linking of clottable blood protein by transglutaminase is thought to pre-date the proteolytic cascade in the evolution of blood coagulation (Lorand et al. 1966, Laki 1972). The use of a transglutaminase in covalent cross-linking of clottable protein is widespread, occurring in all vertebrates (Doolittle et al. 1963) and many invertebrate species (reviewed in Sritunyalucksana and Soderhall 2000, Osaki and Kawabata 2004, Theopold et al. 2004, Jiravanichpaisal et al. 2006). Transglutaminase mediated cross-linking has been well documented in various crustaceans including the lobster *Panulirus interruptus* (Fuller and Doolittle 1971a), the crayfish *Pacifastacus leniusculus* (Kopacek et al. 1993, Hall et al. 1999) and the sand crab *Ovalipes bipustulatus* (Madaras et al. 1981), and results in an \( \varepsilon-(\gamma\text{-glutamyl})\text{lysine} \) cross-link between clotting proteins. Furthermore, the gene encoding *P. leniusculus* transglutaminase is homologous to vertebrate factor XIIIa (Wang et al. 2001). Transglutaminase clotting activity in crustaceans differ from that of vertebrates, in that proteolytic processing of the transglutaminase enzyme is not necessary (Fuller and Doolittle 1971b, Lorand 1972). During clotting, crustacean transglutaminase is released from hemocytes and is activated by \( \text{Ca}^{2+} \) (Sritunyalucksana and Soderhall 2000). Proteolytic activity is involved in assembly of clotting proteins (as shown in *O. bipustulatus*: Madaras 1981; and *Homarus vulgaris* & *Jasus lalandei*: Durliat and Vranckx 1981) and activation of the pro-phenoloxidase pathway (Soderhall 1981) rather than transglutaminase activation.

A fibrin blood clot that has been cross-linked via factor XIIIa is insoluble in urea (Laki 1972). Fibrin blood clot solubility in urea has been used as an indicator that cross-linking via factor XIIIa has occurred. Likewise, polymerized barnacle cement has been shown to be insoluble in 8M urea (Kamino et al. 1996, Naldrett and Kaplan 1997). To date, polymerized barnacle cement has not been rendered fully soluble under any
conditions (Kamino 2006). Disulfide bonds and hydrophobic interactions are thought to account for some of the insolubility of barnacle cement (Naldrett 1993, Kamino et al. 1996, Naldrett and Kaplan 1997, Kamino et al. 2000). In addition to these mechanisms, covalent cross-linking of barnacle cement proteins brought about by a transglutaminase is likely to play a key role in the inherent insolubility of barnacle cement and may explain why a portion of the cement is insoluble even under strong denaturing treatments.

3.4.5 Conclusions

The data presented in this chapter provide clear evidence that barnacle cement polymerization and blood coagulation occur by a similar enzymatic mechanism and strongly support the hypothesis that these two processes were derived from a common ancestral mechanism. Similar to clotted fibrin, polymerized barnacle cement exists as a mesh of tightly interlocking fibrous proteins. Barnacle cement polymerization can be inhibited by anticoagulants including trypsin inhibitor, suggesting the role of trypsin in cement polymerization. Trypsin activity is Ca$^{2+}$ dependent and may be regulated by both an active site trypsin inhibitor and a steric trypsin inhibitor (A2M). The trypsin-like protein in barnacle cement is homologous to bovine pancreatic trypsin in terms of the catalytic region. A direct homologue to human factor XIII (fibrin stabilizing factor, transglutaminase), A1 subunit precursor is present and active in barnacle cement and can mediate covalent cross-linking between cement monomers via transglutamination.

A schematic of barnacle cement polymerization, based on the data presented in this chapter is shown in figure 29. During growth, repair and reattachment, cement is released containing structural precursors, pro-trypsin (inactive) and inactive factor XIII (transglutaminase, contained within hemocytes). At least one, and possibly multiple trypsin-like proteases will be released. Initial pro-trypsin activation may occur in
response to a foreign surface. Trypsin activity, which is Ca$^{2+}$ dependent, will activate cement structural precursors and additional pro-trypsin. Protease inhibitors are likely to regulate trypsin activity. Activated cement structural proteins will form a loose assembly and rearrange with the surface. Covalent cross-linking, brought about by Ca$^{2+}$ activated XIIIa (transglutaminase) will reinforce the cement. Reorganization of activated structural proteins and covalent cross-linking result in a mesh of tightly interwoven fibrous protein. Throughout the cement polymerization process, signal peptides are released which stimulate settlement of barnacle cyprid.
Figure 29: Schematic of barnacle cement polymerization as described in Chapter 3. Red arrows indicate enzymatic activity, green arrow are peptide release and blue arrows show enzymatic regulation.
4. Heritable Defects in the Coagulation Mechanism

4.1 Introduction

Protein coagulation systems are inherently imperfect. Coagulation of protein in biological systems is typically a complex, multicomponent process often involving an enzymatic cascade. A defect in a single component of the system can lead to ineffective or incomplete coagulation. Defective coagulation has been well studied in the context of vertebrate blood coagulation. Deficiency of a single clotting factor within the vertebrate blood coagulation cascade can result in an inability to coagulate blood (Davie 2003). The outcome of this deficiency varies depending on what factor is defective or lacking. For instance, individuals missing factor XII (Hageman factor) often show no signs of an inability to clot, as an alternative mechanism to activate the next step in the cascade is available (Davie et al. 1991). In contrast, individuals with a defective gene for factor VIII (Antihemophilic factor) are unable to clot and often lose large amounts of blood upon minor injury (Lawn and Vehar 1986). Factor VIII plays an essential regulatory role and its absence prevents the propagation of the blood coagulation cascade. Defective blood coagulation has likewise been shown in invertebrates (specifically in crustaceans) with individuals varying in the ability and time required to coagulate blood (Durliat and Vranckx 1983a, b, Jussila et al. 2001). The cause of this variability is not clear, although differences in blood protein levels as well as the number and types of hemocytes have been suggested (Jussila et al. 2001).

As described in Chapters 1 and 2, vertebrate and invertebrate blood coagulation provide a valuable context in which to study the coagulation of barnacle cement. Genetic variability in the morphology of polymerized barnacle cement has been shown (Holm et al. 2005). Altered cement morphology may result from specific differences in the cement
polymerization process, similar to those observed in blood coagulation. Variability in cement morphology is exclusively expressed when barnacles are grown on low surface energy silicone coatings. Barnacles always produce a thin, hard, transparent cement when grown on polystyrene or glass surfaces. In contrast, barnacles grown on low surface energy silicone coatings occasionally produce a thick, gummy, opaque cement, which may be incompletely or improperly cured (Watermann et al. 1997, Berglin and Gatenholm 2003, Wiegemann and Watermann 2003, Holm et al. 2005). A clear difference in cement morphology can be seen between a barnacle with thick, gummy cement (referred hereafter as “gummy”) versus one with hard, thin cement (referred to as “hard”; Figure 30). Gummy cement is noticeably soft and can be several millimeters thick. The percentage of *A. amphitrite* individuals producing gummy cement varies with the type of silicone used, with 31% of individuals showing gummy cement on Veridian®, silicone, a commercial mold-release coating and 18% exhibiting this trait on Silastic T2®, a silicone rubber (Holm et al. 2005).

![Figure 30: The base plate of a hard barnacle (left) and gummy barnacle (right), both *Amphibalanus amphitrite*. Barnacles were grown on a Veridian® silicone coated glass plate. Note the radial canals of the base plate, which are clearly seen for the hard barnacle but cannot be seen for the gummy barnacle.](image-url)
Investigations into the ultrastructure of hard and gummy cement have been conducted. Using scanning electron microscopy and atomic force microscopy, Wiegemann and Watermann (2003) determined that hard cement (left by Balanus improvisus on aluminum foil) was composed of a tightly woven network of globules characteristic of progressive protein cross-linking, whereas gummy cement left by Balanus improvisus on polydimethylsiloxane (PDMS silicone) was composed of a loose network of hydrated adhesive threads. Using electron probe microanalysis, Berglin and Gatenholm (2003) considered calcium content of Balanus improvisus cement. Calcium was found incorporated as calcite in hard cement (barnacles grown on long-chain hydrocarbon polymethylmethacrylate: PMMA), as compared to gummy cement in which calcium could not be detected (barnacles grown on PDMS silicone). A recent scanning electron microscope and energy dispersive X-ray analysis study conducted at Clemson University confirmed the results of Wiegemann and Watermann (2003) and Berglin and Gatenholm (2003) using A. amphitrite (Gohad, N.V., Dickinson, G.H., and A.S. Mount, Unpublished Data).

In addition to dissimilar morphology and chemical content, hard and gummy cement differ in their adhesive properties. The force required to remove a gummy barnacle from a silicone substrate is generally less than that of a hard barnacle (Holm et al. 2005, shown for 1 of 2 silicones used, Wendt et al. 2006). Formation of a thick, gummy cement rather than a thin, hard cement may lead to changes in both adhesive chemistry and base plate mechanics. As described by Chung and Chaudhury (2005) base plate mechanics (flexing) may play a significant role in the release properties of barnacles. In this study, I seek to determine if plate mechanics play a role in the observed differences in adhesive strength between hard and gummy barnacles or if this difference can solely be attributed to differing adhesive chemistry between the two cement phenotypes.
Similar to blood clotting disorders in vertebrates (Lawn and Vehar 1986), the production of gummy cement is a heritable trait (Holm et al. 2005). Using 47 maternal families of *A. amphitrite* bred in the laboratory, Holm et al. (2005) determined the broad-sense heritability of gummy cement production to be 0.46 for barnacles grown on Veridian® coated panels and 0.59 for those on Silastic T2®. Of the 47 maternal families, certain families expressed a high proportion of gummy cement on both silicones, others produced a low proportion of gummy on both silicones while others still produced a high proportion of gummy cement on one surface but not the other (Table 3). In the years since the Holm et al. (2005) study, support of a heritable basis for gummy cement production has been provided through controlled pair-wise matings, which have shown parents with gummy cement to produce offspring with gummy cement, and the ability to generate a second set of families with similar proportions of gummy cement to those shown on table 3 (Orihuela et al., In preparation).

<table>
<thead>
<tr>
<th>Family</th>
<th>Percent Gummy on Veridian</th>
<th>Percent Gummy on T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low on Veridian &amp; T2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Higher on Veridian</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Higher on T2</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>High on Veridian &amp; T2</td>
<td>66</td>
<td>76</td>
</tr>
</tbody>
</table>

The observed phenotypic variation shown for a trait is the sum of the environmental and genetic variance of that trait. Holm et al. (2005) showed that genetic effects play a role in determining cement morphology. It is unclear, however, how this
genetic variation is manifested. As proposed by Holm et al. (2005), gummy versus hard
cement production could result from either: 1) a physical mechanism in which
individuals respond to reduced adhesive strength on silicone in a heritable manner, with
the base plate of certain individuals (gummy) lifting from the surface and hydrated
cement filling the newly formed gap; or 2) a chemical mechanism in which the chemistry
of the cement produced by gummy barnacles differs from that of hard barnacles. The
difference in cement chemistry would be such that interactions with the silicone
substrate (including molecules released from the surface) will interfere with cement
polymerization in gummy barnacles but not in hard barnacles.

Silicone has long been known to interfere with the coagulation of blood proteins,
(Jaques et al. 1946) and is routinely used in clinical applications to prevent the clotting
of isolated blood. Silicone is thought to interfere with the contact dependent reactions
that initiate blood coagulation, which require binding to a negatively charged surface
(Davie and Rantoff 1964). If a chemical mechanism is involved in the production of
alternative cement morphologies, enzymatic and structural components of gummy
cement may be more sensitive to surface charge and surface energy than those of hard
cement. In addition to charge and surface energy, the silicone substrate may be leaching
molecules from the surface (Berglin and Gatenholm 1999, 2003, Meyer et al. 2006,
Rittschof et al. 2007). Leached molecules will come in direct contact with unpolymerized
cement when it is released by the barnacle onto a silicone surface, and therefore could
interfere with cement polymerization. In gummy barnacles, the enzymes and structural
components involved in cement polymerization may be more vulnerable to interference
from leached molecules than are the enzymatic and structural components of hard
barnacles. Support for this hypothesis has been shown by cement polymerization
inhibition assays with anticoagulants (Chapter 3, gel electrophoresis), which show a
greater difference in the intensity of protein bands between inhibitor treatments and control for gummy barnacles than for hard barnacles.

The purpose of this study is to consider gummy versus hard cement production within the context of blood coagulation, determining if chemical differences between hard and gummy cement exist and assessing if specific defects in the cement polymerization process result in the formation of gummy cement. Objectives include: 1) quantify biomechanical properties (composite modulus, base plate thickness, flexural rigidity and cement hydration) of hard and gummy barnacles to determine if differences in adhesive strength can be solely attributed to differences in cement chemistry or if differences in base plate mechanics (flexing) are also involved; 2) assess if chemical differences exist between cement from hard and gummy barnacles in terms of cement components and composition (including the concentration of specific enzymes identified in Chapter 3 to be involved in cement polymerization, trypsin and transglutaminase), cement polymerization time course, and polymerized cement chemistry; and 3) consider the potential for molecules released from silicone to interfere with cement polymerization by evaluating the presence and identity of leached molecules. The production of an incompletely or improperly cured cement within a complex multicomponent coagulation system will be discussed.
4.2 Materials and Methods

4.2.1 Barnacle Larval Culture, Settlement and Maintenance

The barnacle Amphibalanus amphitrite (= Balanus amphitrite) (Pitombo 2004) was used for this study. Barnacle larval culture and settlement was conducted at the Duke University Marine Laboratory in Beaufort, North Carolina, following Rittschof et al. (1984a). Barnacle larvae were settled on 7.6 x 15.2 x 0.64 cm glass panels coated with silicone (Dow Corning Silastic T2® or International Veridian®) and maintained in the laboratory as described by Holm et al. (2005).

For experiments conducted at the Naval Research Laboratory (NRL), barnacles on silicone coated panels were transported to the Naval Research Laboratory in Washington, DC after 5 weeks of growth. While at NRL, panels were kept in individual plastic containers filled with artificial seawater (32 ppt, Instant Ocean® in doubly distilled water, aerated overnight before use). Artificial seawater was changed twice a week. At NRL, barnacles were fed with 10 ml dense Artemia sp. (Sanders, Morgan, UT, hatched from approximately 1 teaspoon cysts in 1 L seawater) every day for 10 weeks and then every other day thereafter.

4.2.2 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Chemicals were of the highest grade available. Product numbers are provided the first time a chemical is listed.
4.2.3 Hard vs. Gummy: Biomechanical Properties

4.2.3.1 Composite Modulus, Base Plate Thickness and Flexural Rigidity

4.2.3.1.1 Definitions

Definitions are based on Denny (1988) and Symon (1971):

Compliance: The compressibility of a material, calculated when a force is applied to a material as a ratio of the distance the material is deflected (displacement) to the magnitude of the applied force.

Composite Modulus: Elastic modulus (Young’s modulus) is a measure of the stiffness or deformability of a material and is defined as the ratio of stress to strain. Stress is the force per unit area acting on a material and strain is the magnitude of deformation caused by a stress. The elastic modulus of a material such as rubber would be very low, whereas that of diamond would be very high. Composite modulus is used in this study since base plate modulus measurements will reflect both proteinaceous cement and the calcified base plate.

Flexural Rigidity: Flexural rigidity is a measure of the tendency for a rigid object (in this case a plate) to flex or bend when a force is applied. It will be dependent on the modulus of the object, the dimensions of the object (the diameter and thickness for a plate) and the Poisson’s ratio of the object.

4.2.3.1.2 Experimental Procedure

For mechanical testing of hard vs. gummy barnacles, a punch test apparatus was constructed, which consisted of a 1000 gram load cell (GSO, Transducer Techniques, #GSO-1K) attached to a vertically mounted linear positioning stage (Aerotech, #ATS50). A cylindrical punch mounted on the load cell was lowered at 0.005 mm sec\(^{-1}\) toward an inverted, rigidly mounted barnacle. Two barnacle supports were fabricated.
Each support consisted of two steel plates 1.6 mm in thickness (Kimball Physics, Inc.) glued to a partially hollowed brass cylinder (~25 mm diameter, 30 mm height) using cyanoacrylate adhesive. The steel plates of the larger support had a central hole of 10 mm diameter, while the smaller design had a 6.5 mm diameter opening. Two punches were used in conjunction with the different supports: a 5.10 mm diameter punch head (Transducer Techniques, #ALS-08) for larger barnacles, and a 4.10 mm diameter punch head for the smaller. Labview® (National Instruments) data acquisition program was used to monitor load cell voltage readings and simultaneously control stage motion.

Compliance measurements were made on barnacles of two different age groups, 3 months old (Set #1) and 14 months old (Set #2), from barnacles settled at the same time (same larval cohort). Barnacles were affixed securely to the steel supports using a methyl methacrylate resin (Teets Denture Material) around the barnacle that cured for 20 to 60 min before testing. Experiments were initiated with the punch out of contact with the barnacle base. Data were collected as the punch came into contact, pushed into the barnacle and pulled back out of contact. The slope of the linear regime of the force curve, in mm N⁻¹, corrected for instrument compliance, provided a measure of the barnacle base plate compliance.

In many individuals the gummy cement phenotype is incompletely expressed (gummy cement on a portion, but not the entire base plate: Holm et al. 2005). Before testing, the amount of gummy cement was empirically approximated and recorded as a percentage of the total base plate area. For this study, barnacles were qualitatively categorized “hard” if ≤ 30% of the base plate was covered in opaque, soft cement (as determined by visual inspection) and “gummy” if >30% of the base plate was covered in opaque, soft cement. Note, the definition of hard versus gummy is slightly different than that used in biochemical studies (described below) due to barnacle availability at the Naval Research Laboratory where biomechanical testing was conducted.
Classification as hard or gummy cement was conducted immediately upon release since the appearance of soft cement changes as the cement is exposed to air. Use of barnacles with a portion, but not their entire base plate covered in gummy cement allowed the determination of biomechanical properties along a gradient of increasing gummy cement coverage. The base plate diameter was measured using calipers along parallel and perpendicular axes to the opercular opening. After punch testing, the barnacle base was fractured by hand, and a section from the center (where the punch contact was made) was removed and measured for thickness with digital calipers to the nearest 10 μm. Care was taken not to compress the gummy adhesive during the thickness measurement.

Composite modulus and flexural rigidity values for each barnacle were calculated based on base plate compliance, thickness and radius (Timoshenko 1970). For these calculations, fixed edges and a uniform load over a concentric circular area were assumed (Kutz 1998). The base plate Poisson’s ratio was estimated at 0.3 based on CaCO₃ as the primary constituent of the calcified base plate (Bourget 1987, Rodriguez-Navarro et al. 2006). Statistical analysis of data was conducted using SigmaStat® V. 3.10.0.

The experimental procedure was modified slightly between measurements on a group of 18 three month old barnacles (Set #1) and another group of 25 fourteen month old barnacles (Set #2). The three month old barnacles (Set #1) were released from their silicone substrates before mounting in the dental cement, exposing the base plate to air during the curing process before testing. Due to a concern that some dehydration of the base plate might affect the measurements, the fourteen month old barnacles were not released from the substrate until immediately prior to testing. Instead, barnacles were coated in methyl methacrylate while still attached to silicone panels, and the substrate was inverted and clamped to a manual stage. The coated barnacle was then lowered
onto the support using the stage, cured in air for 25 min, removed from the silicone, and immediately tested. This technique reduced the chance for mechanical modification of gummy cement by exposure to air.

### 4.2.3.2 Water Content of Gummy Cement

Water content of gummy cement was determined as the change in mass over time in air. Barnacles were removed from their silicone substrate and the base plate was inspected for gummy cement. For barnacles with 100% of their base plate covered in gummy cement, the cement layer was immediately cut from the base plate using a scalpel and placed into two approximately 1 cm², clean aluminum foil dishes. The cement sat in air at room temperature and humidity (21.1°C, 70% humidity). Mass of the cement was recorded at 0, 5, 10, 15, 20, 30, 45, 60 and 960 min. To assess water vapor deposition during the course of the experiment, two empty aluminum foil dishes were weighed on the same time intervals as cement samples. Two additional dishes containing gummy cement were heated at 128°C for 24 hrs.

### 4.2.4 Hard vs. Gummy: Biochemical Properties

#### 4.2.4.1 Unpolymerized Barnacle Cement

For studies utilizing unpolymerized barnacle cement, unpolymerized cement droplets were obtained by hand using a method inspired by Cheung et al. (1977). Cement production is continuous throughout a barnacle’s life (Saroyan et al. 1970), which makes the collection of unpolymerized cement possible. Barnacles were gently removed from a silicone foul-release substrate using a dissecting needle (Hamilton Bell Co. Inc., Montvale, NJ). Barnacles whose base plate broke upon removal were discarded. Immediately following release from silicone, barnacles were classified by cement type (hard or gummy). For studies on cement biochemical properties, barnacles were
considered “hard” if there was no opaque, soft cement on the base plate (as determined by visual inspection and gentle probing with a dissecting needle) and were considered “gummy” if >90% of the base plate was covered in opaque, soft cement. Barnacles showing intermediate levels of opaque cement coverage were not used for biochemical studies. Only barnacles whose bases were completely in contact with the silicone panel were utilized for this study; barnacles growing in contact with other barnacles or on the edge of the panels were not used. Classification as hard or gummy was conducted immediately upon release since the appearance of soft cement changes as the cement is exposed to air.

Following classification, all shell plates (including the base plate) were gently cleaned in deionized water with a cotton swab. Barnacles were then dried with a Kimwipe® and sat in air on a paper towel for 3 hrs. Allowing time for the barnacles to dry is essential for the formation of defined cement droplets. To stimulate release of cement, the periphery of the base plate (where cement is normally released during growth; the junction between the base plate and parietal plates) was gently pricked in an outward direction with a dissecting needle. Opening the cement channels by removing previously polymerized/calcified cement allows for 1-2 μl droplets to form, which can be taken up with a 0.5 – 10 μl pipettor with a micro tip. Very gently squeezing the barnacle between the thumb and finger (compressing the base plate towards the operculum) increased cement volume. After cement collection, unattached barnacles were maintained in 10.5 cm glass finger bowls for up to two months, with hard and gummy held in separate finger bowls. To prevent strong adhesion to the glass during this time, each barnacle was pushed gently to a different location in its finger bowl daily. Barnacles were used on average once per week for cement collection as described above.
4.2.4.2 Total Protein Concentration

A Coomassie protein assay (Bradford 1976) was used to determine the total protein concentration in unpolymerized cement from hard and gummy barnacles. Total protein assays were conducted using Coomassie Protein Reagent (Pierce Chemical, #23200). 0.5 μl unpolymerized cement was taken from each barnacle and immediately added to 15 μl deionized water, vortexed and placed on ice. When all samples were collected, each sample was distributed in 3, 5 μl aliquots in a 96 well plate containing 250 μl Coomassie reagent. A BSA standard curve (0, 31.25, 62.5, 125, 250, 500 and 1000 μg ml⁻¹) was run with cement samples. Samples were read at 595 nm on a Molecular Devices SpectraMax® 190 spectrophotometer. Total protein was quantified for 25 hard barnacles and 29 gummy barnacles. Statistical analysis of proteins assays was conducted using SigmaStat® V. 3.10.0.

4.2.4.3 SDS-PAGE

Barnacle cement proteins polymerize rapidly upon removal from the barnacle base and therefore rapid denaturing of the cement proteins is necessary to prevent polymerization. For SDS-PAGE, reducing sample buffer containing 10% (w/v) SDS and 5% (v/v) β2-mercaptoethanol (Modified from Laemmli 1970) was added directly to unpolymerized cement and samples were heated at 100°C for 4 min. Reducing sample buffer was added in excess (80% total volume rather than 50%) to prevent polymerization of cement proteins. 1-3 μl unpolymerized cement was loaded on each lane. Although the volume of cement varied between gels depending on the application, all lanes within a gel contained the same initial volume of unpolymerized cement. Barnacle cement contains, on average, 10.3 μg protein per microliter, and therefore the protein content in 1 μl cement was sufficient to visualize protein bands with Coomassie Blue stain. Samples were run on a 4-20% gradient gel (Pierce Precise Precast Protein Gel,
Product #25224: 12 lane, 30 μl or #25244: 15 lane, 25μl) along with molecular weight markers (Novagen Trail Mix 10 – 225 kDa Protein Markers, #70980-3) at 40 volts for 15 min and then at 100 volts for 1 hr.

Following electrophoresis, gels were stained overnight with Coomassie Blue stain (0.25% Coomassie Brilliant Blue R-250 (BioRad Electrophoresis grade, #161 0400), 7.5% acetic acid, 5.0% methanol), then destained in a solution of 25% methanol, 7.5% acetic acid for 30 min and finally destained in several changes of 7.5% acetic acid for 24-48 hrs. Gels were photographed after destaining with a digital camera. To allow direct comparison of band intensity among gel lanes, each gel lane was analyzed for pixel intensity using Scion Image® V. Alpha 4.0.3.2 and plotted using SigmaPlot® V. 9.0.

4.2.4.4 Barnacle Families: SDS-PAGE

SDS-PAGE (reducing conditions) was conducted on individuals from families of barnacles with a known proportion of hard versus gummy barnacles (see Table 3). The F1 generation of these families was bred in July 2004 (Holm et al. 2005) and the F4 generation was used for this study. For each SDS-PAGE assay, 2 μl unpolymerized cement was taken from a hard and a gummy individual from each of four families:

1) 3-1: low proportion “gummy” on Veridian & T2
2) 1-15: high proportion “gummy” on Veridian, low proportion “gummy” on T2
3) 3-12: low proportion “gummy” on Veridian, high proportion “gummy” on T2
4) 2-2: high proportion “gummy” on both Veridian & T2

Reducing sample buffer was immediately added to unpolymerized cement and samples were heated, run and analyzed as described previously. Barnacle family SDS-PAGE assays were conducted on three separate occasions.
4.2.4.5 Polymerization Time Course: SDS-PAGE

SDS-PAGE was conducted to determine if and in what ways the number and intensity of resolvable proteins bands changed as cement polymerized. Unpolymerized cement was incubated in air for a given amount of time, then subjected to denaturing treatment and run on SDS-PAGE (reducing conditions). 2 μl unpolymerized cement was taken from both hard and gummy barnacles, placed in a microfuge tube and allowed to polymerize in air for 0, 2, or 15 min before denaturing treatment. Each incubation time was run in duplicate for both hard and gummy cement. After the 0, 2 or 15 minute incubation time, reducing sample buffer (composed as described above) was added to cement, and samples were heated at 100°C for 4 min. After all cement samples had been denatured, electrophoresis was run and analyzed as described previously. SDS-PAGE polymerization assays were conducted three times at room temperature. A fourth polymerization assay was conducted using 1 μl cement, with cement on ice during polymerization.

4.2.4.6 Polymerization Time course: FTIR

Fourier Transform Infrared Spectroscopy (FTIR) was performed using Attenuated Total Reflectance (ATR). FTIR was conducted on a Nicolet Magna-IR 750 Spectrometer with a DTGS KBr detector. Germanium ATR crystals (Harrick Model # EJ2122, 45°, 50 x 10 x 2 mm) were used for IR experiments. ATR crystals were reused up to three times, with each crystal cleaned thoroughly with ethanol between assays. The ATR crystal was mounted in a stainless steel Harrick Horizon® multiple reflection ATR accessory, with a water-tight gasket. Prior to placement of barnacles onto the ATR crystal, at least 15 background spectra of the clean ATR crystal were taken over the course of 30 min. All background spectra for each assay were averaged to serve as a baseline for spectral analysis.
To obtain IR spectra of barnacle secondary cement, 5-7 hard or gummy barnacles were removed from a Silastic T2® silicone coated panel using a dissecting needle, the shell plates of the barnacles were dried with a Kimwipe® and barnacles were immediately placed on an ATR crystal, on which they would secrete cement. The usable surface area of the ATR crystal measured 50 x 8 mm, which allowed for placement of 5–7 barnacles, depending on barnacle size. Three IR assays with hard barnacles and three IR assays with gummy barnacles were conducted.

Spectra acquisition began immediately after placement of barnacles on the ATR crystal. Spectra were taken summing either 32 or 16 scans at a resolution of 8 wavenumbers. Spectra were taken every minute for the first 15 min after placement, every 3 min for the next 45 min and every 10 – 15 min for the next 8 hrs. Barnacles were left to reattach in air for up to 23 hrs or until no change was observed in spectra. After this time period, a small amount (1-2 ml) of artificial seawater was added to the trough of the ATR element. Enough seawater was added to cover the barnacle base plates, but not to cover the operculum. A glass microscope slide was placed over the trough of the ATR element to minimize evaporation. Spectra were acquired every 10 – 15 min for 3.5 – 4 hrs while the barnacles’ base plates were covered in seawater.

After 3.5 – 4 hrs in seawater, the ATR element was removed from the spectrometer and barnacles were carefully removed from the ATR crystal using forceps. Seawater was poured off the Ge crystal and the crystal was rinsed with a small amount of deionized water and gently blown dry with air. Cement left by reattaching barnacles could clearly be seen on the ATR crystal. The ATR element with residual cement was placed back on the spectrometer. Spectra of residual cement were taken every 15 min for 1 hr or until no changes between spectra were observed.
4.2.4.7 Trypsin Immunoreactivity

Trypsin immunoreactivity towards bovine pancreatic trypsin antibody was assessed with dot blotting. Dot blots of barnacle cement droplets were conducted by placing 0.5 μl of unpolymerized cement and control solutions (Positive control: bovine pancreatic trypsin, TPCK treated, #T1426, at 1 mg ml⁻¹, Negative control: BSA, #B4287 at 1 mg ml⁻¹, and porcine fibrinogen, fraction I, #F2629, at 0.5 mg ml⁻¹) directly onto a PVDF membrane (Millipore Immobilon P™, 0.2 μm pore size, #ISEQ 081 00) that had been activated with methanol and rinsed with deionized water. PVDF membranes with cement and control solutions were allowed to dry and stored at 4°C overnight. Immunostaining was conducted the following day.

Two techniques were used for immunostaining of dot blots, a standard method and a rapid detection method. Cement (from hard and gummy barnacles) and all controls described above were assessed using both methods. For the standard method, the PVDF membrane was reactivated in methanol, blocked for 2 hrs in 5% non-fat dry milk, then incubated with primary antibody for 2 hrs and secondary antibody for 1 hr with 3 x 10 min washes with TBS (containing 0.5% NFDM and 0.1% Tween® 20) in between each step. The rapid immunodetection method (Millipore 2005) is based on the inability of antibodies to bind to the hydrophobic surface of the PVDF membrane. The PVDF membrane was not activated and was not blocked. The membrane was incubated in primary antibody for 1 hr, then secondary antibody for 30 min with 3 x 5 min washes with TBS between steps.

For both methods, rabbit polyclonal antibody to full length bovine pancreatic trypsin (1:10,000 dilution) was used as the primary antibody (Novus Biologicals #NB 600-1277). Goat anti-rabbit polyclonal antibody (1:20,000 or 22,500 dilution), conjugated to alkaline phosphatase was used as secondary antibody (Novus Biologicals
Horseradish peroxidase conjugated secondary antibodies were avoided due to endogenous peroxidase activity of barnacle cement. Antibodies were detected using a BCIP/NBT substrate (Vector Laboratories #SK5400). Control staining for non-specific binding was conducted by incubating membranes in secondary antibody only.

### 4.2.4.8 Trypsin Activity

Trypsin activity can be quantified spectrophotometrically based on tryptic cleavage of arginine esters. BAPNA (Nα-Benzoyl-DL-arginine 4-nitroanilide; Arcos Organics #227740010) was used as a trypsin substrate and prepared at 0.044% (w/v) by first dissolving BAPNA in DMSO (1% v/v) and then adding 50 mM Tris buffer, pH 8.0. Reaction conditions (pH, incubation temperature, buffer concentration) followed Dougherty (1996) who optimized reaction conditions for general protease activity in *Chthamalus fragilis* unpolymerized cement.

To assay trypsin activity, 6 μl unpolymerized cement was added to 800 μl BAPNA solution. Samples were vortexed and incubated at 37°C for 1 hr. Two controls were run along with cement samples: 1) 6 μl Tris buffer (50 mM, pH 8.0) with 800 μl BAPNA solution to account for background hydrolysis of BAPNA, and 2) 6 μl unpolymerized cement with 800 μl Tris buffer (50 mM, pH 8.0, *without* BAPNA) to account for any absorbance contribution of unpolymerized cement unrelated to hydrolysis of BAPNA. Control samples were vortexed and incubated at 37°C for 1 hr. Following incubation, all samples were centrifuged at 9000 rpm for 10 min in a Fisher Scientific MicroD centrifuge. Samples were transferred to a quartz semi-micro cuvette (Starna Cells #9-Q-10) and absorbance at 405 nm (referenced to Tris buffer alone) was read on a Hewlett Packard 8451A diode array spectrophotometer. Samples were staggered in ~8 sample groups (each with controls) so that all samples could be read within 10 min of centrifugation.
Calculation of trypsin activity was based on a trypsin standard curve. The standard curve consisted of 1.85 $10^{-5}$, 9.81 $10^{-6}$, 4.63 $10^{-6}$, 2.31 $10^{-6}$, 1.16 $10^{-6}$, 5.78 $10^{-7}$, 2.89 $10^{-7}$ and 0 BAPNA units ml$^{-1}$ trypsin, prepared immediately prior to use with porcine pancreatic trypsin (Type II-S, #T7409). 6 μl of each standard was combined with 800 μl BAPNA solution, samples were vortexed and incubated at 37°C for 1 hr, centrifuged at 9000 rpm for 10 min, and absorbance was read as described above. Absorbance values for barnacle cement samples were adjusted by subtracting the average absorbance of both controls listed above (BAPNA only and unpolymerized cement in buffer without BAPNA). Using adjusted barnacle cement absorbance values, trypsin activity was extrapolated based on the standard curve. Trypsin activity was calculated for 10 hard and 10 gummy individuals.

4.2.4.9 Transglutaminase Activity

Transglutaminase activity of unpolymerized cement was assessed using a transglutaminase assay kit (Sigma-Aldrich #CS1070). Assays were based on the reaction of transglutaminase with a cadaverine coated 96-well plate. 1 μl unpolymerized cement was used for each test well. Cement was first added to assay buffer in a microfuge tube and centrifuged. Cement in assay buffer was held on ice until all cement samples had been collected, at which time assay buffer containing cement was transferred to plate wells. Blanks (no enzyme present) and positive control (2 milliunits ml$^{-1}$ transglutaminase from guinea pig liver), each in triplicate, were run along with barnacle cement samples. Since quantification of transglutaminase was based on peroxidase conjugated streptavidin, and endogenous peroxidase activity has been shown for barnacle cement (Chapter 2), OD$_{450}$ values were corrected for barnacle endogenous peroxidase activity. 1 μl of cement in assay buffer from each individual was run in wells both with and without streptavidin-peroxidase added. The mean OD$_{450}$ of
blank wells without streptavidin-peroxidase (background absorbance) was subtracted from the OD$_{450}$ of barnacle cement wells without streptavidin-peroxidase to obtain an absorbance value for endogenous peroxidase. The peroxidase correction for each individual was subtracted from the cement well run with streptavidin-peroxidase for that individual to obtain a peroxidase corrected OD$_{450}$ value.

4.2.5 Release of Molecules from Silicone Substrates

Coupled gas chromatography-mass spectrometry was used to assess the presence and identity of molecules released from silicone foul release substrates. Two silicone substrates were analyzed, Dow Corning Silastic T2® and International Veridian®, both of which were made for Holm et al. (2005). Silicone panels were prepared in the spring of 2004 and used as a barnacle growth substrate until they were analyzed in December 2006.

Chemical analyses were conducted at the Institute for Chemistry and Biology of the Marine Environment (ICBM; Oldenburg, Germany). Extraction of leachable molecules from silicone was accomplished by added a 30 µl droplet of methanol to the silicone surface for 30 seconds. Samples were directly analyzed (undiluted) by coupled gas chromatography-mass spectrometry. A WCOT VF-5ms capillary column (Varian, USA) (30 m x 0.25 mm x 0.25 µm film thickness) was employed on a Varian 3900 gas chromatograph equipped with a Saturn 2100T (Varian, USA) ion trap mass selective detector. Samples were injected in splitless mode with an inlet pressure of 72 kPa. The injection port and the interface were held at 260°C. The gas chromatograph was held at 70°C for 1 min and ramped at 15°C min$^{-1}$ to 150°C, 20°C min$^{-1}$ to 250°C and held at this temperature for 2 min. Finally, the column was cleaned at 320°C for 1 min. Helium was used as the carrier gas. The mass selective detector was operated in scan mode (m/z 10-
The electron impact ion-spectra of silicone extract components were compared with entries in the NIST mass spectral library (NIST V. 2005).
4.3 Results

4.3.1 Hard vs. Gummy: Biomechanical Properties

4.3.1.1 Composite Modulus

The base plate composite modulus for 3 and 14 month old hard and gummy barnacles was calculated based on base plate compliance measurements. Composite modulus values between hard and gummy barnacles were significantly different as shown by two-way ANOVA (p < 0.001) with age and cement type as factors (Table 4, Figure 31). Barnacle age was not significant and there was no interaction between year and cement type (age x cement). The hydrated barnacle data (14 month old barnacles, mounted in the testing apparatus before removal from silicone to minimize exposure of cement to air) resulted in an average modulus of 2.9 GPa (SEM ± 0.7) for gummy barnacles, and an average modulus of 6.5 GPa (SEM ± 0.6) for hard barnacles (Figure 31).

Figure 31: Composite modulus for three and fourteen month old hard and gummy barnacles. Gummy is defined as >30% of the base plate covered in compliant, opaque cement. Groups marked with A and B are significantly different (Holm-Sidak multiple comparisons: p < 0.05).

Table 4: Two-way ANOVA for modulus. * indicates significance at p < 0.001.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
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<td>10.100</td>
<td>10.100</td>
<td>1.932</td>
<td>0.172</td>
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<tr>
<td>Cement</td>
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<td>80.737</td>
<td>80.737</td>
<td>15.448</td>
<td>&lt; 0.001*</td>
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<tr>
<td>Year x Cement</td>
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<td>4.948</td>
<td>0.947</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Total</td>
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<td>310.476</td>
<td>7.392</td>
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Table 5: Composite modulus least squared means.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hard, 2006</em></td>
<td>12</td>
<td>4.749</td>
<td>0.660</td>
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<tr>
<td><em>Gummy, 2006</em></td>
<td>6</td>
<td>2.578</td>
<td>0.933</td>
</tr>
<tr>
<td><em>Hard, 2007</em></td>
<td>14</td>
<td>6.484</td>
<td>0.611</td>
</tr>
<tr>
<td><em>Gummy, 2007</em></td>
<td>11</td>
<td>2.885</td>
<td>0.689</td>
</tr>
</tbody>
</table>

4.3.1.2 Base Plate Thickness

Barnacles with a greater proportion of their base plate covered in gummy cement tended to have a thicker base plate (Figure 32). Base plate thickness was measured on hand fractured base plates to the nearest 10 μm with a digital caliper and care was taken not to compress the gummy cement. Regression of base plate thickness on the percent of the base plate covered in gummy cement was significant (One-way ANOVA: p = 0.006).
Figure 32: Thickness of the base plate (μm) versus the percentage of the base plate covered in opaque, gummy cement. Linear regression is statistically significant (one-way ANOVA: p = 0.006).

4.3.1.3 Base Plate Flexural Rigidity

Base plate flexural rigidity was calculated based on base plate compliance measurements for 3 and 14 month old hard and gummy barnacles. A two-way ANOVA was used to analyze flexural rigidity data, with age (3 or 14 months) and cement type (hard or gummy) as factors. Neither age nor cement type showed a significant difference between test groups (Figure 33, Table 6). There was no interaction between factors (year x cement; Table 6). Flexural rigidity least squared means for hard and gummy barnacles from both age groups are shown in table 7. The average flexural rigidity of all barnacles was 0.0020 Nm (SEM ± 0.0003).
Figure 33: Flexural rigidity for 3 and 14 month old hard and gummy barnacles. Gummy is defined as >30% of the base plate covered in compliant, opaque cement. Groups are not significantly different.

Table 6: Two-way ANOVA for base plate flexural rigidity.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
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<tbody>
<tr>
<td>Year</td>
<td>1</td>
<td>5.50 E-7</td>
<td>5.50 E-7</td>
<td>0.195</td>
<td>0.661</td>
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<tr>
<td>Cement</td>
<td>1</td>
<td>3.16 E-6</td>
<td>3.16 E-6</td>
<td>1.122</td>
<td>0.296</td>
</tr>
<tr>
<td>Year x Cement</td>
<td>1</td>
<td>1.25 E-6</td>
<td>1.25 E-6</td>
<td>0.444</td>
<td>0.500</td>
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<tr>
<td>Residual</td>
<td>39</td>
<td>2.82 E-6</td>
<td>2.82 E-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>2.76 E-6</td>
<td>2.76 E-6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7: Flexural rigidity least squared means.

<table>
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<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hard, 2006</td>
<td>12</td>
<td>0.0022</td>
<td>0.0005</td>
</tr>
<tr>
<td>Gummy, 2006</td>
<td>6</td>
<td>0.0020</td>
<td>0.0007</td>
</tr>
<tr>
<td>Hard, 2007</td>
<td>14</td>
<td>0.0023</td>
<td>0.0004</td>
</tr>
<tr>
<td>Gummy, 2007</td>
<td>11</td>
<td>0.0014</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

4.3.1.4 Water Content of Gummy Cement

The change in mass of cement over time in air, presumably due to water loss, for gummy barnacles is shown in figure 34. The majority of water loss occurred after 30 min in air (after the time scale relevant to mechanical testing for modulus). Water loss over 960 min was considerable (up to 87.5%). Mass change of empty aluminum foil dishes was negligible over the full course of the experiment. Mean mass loss for samples heated to 128°C for 24 hrs was 77.4% (SEM ± 1.4).
Figure 34: Gummy barnacle cement mass change over time, in air at room temperature and humidity. The cement layer was removed with a scalpel and placed in clean, 1 cm$^2$ aluminum dishes. Empty dishes were weighed as control.
4.3.2 Hard vs. Gummy: Biochemical Properties

4.3.2.1 Total Protein Concentration

Total protein concentration (as determined by Coomassie protein assay on un polymerized cement) differed significantly between hard and gummy barnacles at $\alpha = 0.1$ (t-test: $t = 1.86; df = 52; p = 0.069$; Figure 35), but did not differ significantly at the 0.05 significance level. Mean total protein concentration for hard barnacles was 8.8 $\mu g \mu l^{-1}$ (SEM ± 0.9) and ranged from 0.75 – 18.8 $\mu g \mu l^{-1}$ whereas for gummy barnacles mean total protein concentration was 11.6 $\mu g \mu l^{-1}$ (SEM ± 1.1), ranging from 2.8 – 29.6 $\mu g \mu l^{-1}$.

Figure 35: Mean protein concentration (± SE) for un polymerized cement from hard ($n = 25$) and gummy barnacles ($n = 29$). Protein assay was conducted using a Coomassie assay. t-test: $p = 0.069$. 

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4.3.2.2 SDS-PAGE of Unpolymerized Cement

SDS-PAGE under reducing conditions of unpolymerized cement from a hard and a gummy barnacle is shown in figure 36. To allow detailed comparison of band intensity among gel lanes, Coomassie Blue stained SDS-PAGE gels were photographed, each gel lane was analyzed for pixel intensity and the data were plotted. The most consistent difference between SDS-PAGE of cement from hard versus gummy barnacles is the overall intensity of protein bands within the 150 – 35 kDa range. For most gels run, bands in the 150 – 35 kDa region were more intense for gummy barnacles than for hard barnacles. Proteins in the 125 – 60 kDa range often (but not always) appear as doublet bands for gummy barnacles and as single bands for hard barnacles, as shown in figure 36. The intensity of protein bands at 200 and 30 kDa was usually similar between hard and gummy barnacles.
Figure 36: SDS-PAGE of unpolymerized cement (2μl) from hard and gummy barnacles. Reducing sample buffer was added quickly to unpolymerized cement after removal from the barnacle base. A typical gel lane for hard (left) and gummy (right) is shown along with protein band intensities. Each peak is a band on the SDS-PAGE gel. After staining with Coomassie Blue, the gel was photographed, each lane was digitally analyzed for pixel intensity and the data plotted.

4.3.2.3 SDS-PAGE: Unpolymerized Cement From Barnacle Families

SDS-PAGE (reducing conditions) was conducted on hard and gummy individuals from four families (protein band intensities: Figure 37). The F₁ generation of each family differed in the proportion of gummy individuals, as shown in Table 3. The number and intensity of protein bands varied with the family’s propensity to develop gummy cement. Within the 150 – 35 kDa region, families that had a higher proportion of gummy individuals showed more intense protein bands. The difference in protein band intensity between hard and gummy individuals was greater for mixed families (high proportion of gummy individuals on one silicone surface but not the other) than for un-
mixed families (either high or low proportion gummy on both silicone surfaces). A similar overall pattern emerged for replicate SDS-PAGE conducted on different individuals from the same families.

Figure 37: Protein band intensities for SDS-PAGE gel: unpolymerized cement (2 μl) from hard and gummy individuals representing four families. Reducing sample buffer was added quickly to unpolymerized cement after removal from the barnacle base. Each peak is a band on the SDS-PAGE gel. After staining with Coomassie Blue, the gel was photographed, each lane was digitally analyzed for pixel intensity and the data plotted.
4.3.2.4 Polymerization Time Course: SDS-PAGE

Barnacle cement polymerizes rapidly in air. As polymerization proceeds, the number and intensity of resolvable bands decreases (Figure 38). Protein bands in the 150–35 kDa region show the most significant changes over time. Bands at 200 and 30 kDa do change in intensity over time, but observed changes are generally less significant than those seen in the 150–35 kDa regions. In general, polymerization occurred more rapidly in cement from gummy barnacles than in cement from hard barnacles when the polymerization time course assays were conducted at room temperature (based on the magnitude of change in intensity over time for proteins within the 150–35 kDa, i.e. figure 38). For gummy barnacles, clear changes in the number and intensity of protein bands occurred after 2 min polymerization time, whereas for hard barnacles changes were not observed until polymerization had proceeded for 15 min. When a polymerization time course assay was conducted with cement polymerizing on ice, the number and intensity of protein bands did decrease over time, but polymerization proceeded at approximately the same rate for cement from hard and gummy barnacles.
Figure 38: Protein band intensities for SDS-PAGE gel: polymerization time course. Barnacle cement (2 μl) was incubated in air at room temperature for 0, 2 or 15 min before reducing sample buffer was added. Each peak is a band on the SDS-PAGE gel. After staining with Coomassie Blue, the gel was photographed, each lane was digitally analyzed for pixel intensity and the data plotted.
4.3.2.5 Polymerization Time Course: FTIR

The polymerization time course, as shown by FTIR of whole barnacles as they release cement and as the cement polymerizes in air, differs significantly between hard and gummy barnacles (Figures 39 & 40). Peaks in the amide regions, which are associated with protein absorption, begin to increase earlier in the course of polymerization for gummy barnacles than for hard barnacles. As polymerization proceeds, however, the overall intensity of amide regions becomes greater for hard barnacles than for gummy barnacles. Certain regions (3079 – 2853 cm\(^{-1}\) in particular) are distinctly different between hard and gummy barnacles. Peaks within this region are likely –CH\(_3\) and –CH\(_2\) stretching vibrations. These peaks begin to grow in intensity at 50 min for hard barnacles but are never observed for gummy barnacles.

For hard barnacles, the initial (0 min) spectra is dominated by a broad peak at 3378 cm\(^{-1}\) (–OH stretch) and a sharp peak at 1642 cm\(^{-1}\) (bending mode of liquid water). The intensity of both the –OH stretch at 3378 cm\(^{-1}\) and the water bending mode at 1642 cm\(^{-1}\) increase dramatically over the first 7 min of polymerization. The amide A region (3284 cm\(^{-1}\)) increases in intensity, relative to the –OH stretch at 3378 cm\(^{-1}\), after 7 min indicating absorption of protein to the surface and exclusion of water. The amide I, II and III regions (1750 – 1200 cm\(^{-1}\); marked on figure 40), associated with protein absorption, begin to increase in intensity and become defined after 7 min for amide I and II and after 25 min for amide III. Likewise, after 7 min the amide II peak (1541 cm\(^{-1}\)) increases in intensity relative to the amide I peak (overlapping the bending mode of liquid water ~1640 cm\(^{-1}\)) further indicating absorption of protein to the surface and exclusion of water.

The initial spectra (0 min) for gummy barnacles is significantly different than that of hard barnacles. For gummy barnacles, the initial spectra (0 min) is dominated by a
very intense $\text{--OH}$ stretch at 3883 cm$^{-1}$ and intense water bending mode around 1640 cm$^{-1}$. In contrast to hard barnacles, these peaks immediately begin to decrease in intensity (rather than increasing and then decreasing as seen in hard). The amide A region (3284 cm$^{-1}$) begins to grown in intensity relative to the $\text{--OH}$ stretch at 3378 cm$^{-1}$ after 2 min, but is not well defined until 25 min. Peaks in the amide II and III region begin to become defined and grown in intensity after 2-7 min, rather than after 7-25 min as seen in hard barnacles. Relative to the amide I peak (overlapping the bending mode of liquid water $\sim$1640 cm$^{-1}$), the amide II peak (1541 cm$^{-1}$) gradually increases in intensity after 2 min indicating absorption of protein to the surface and exclusion of water.

![Figure 39: All FTIR spectra for hard (left) and gummy (right) barnacle cement polymerization in air. Barnacles were allowed to reattach to an ATR crystal. Note, absorbance and times scales are not equivalent between hard and gummy.](image)
Figure 40: A subset of the FTIR spectra shown in figure 39, for hard (left) and gummy (right) barnacle cement polymerization in air. Barnacles were allowed to reattach to an ATR crystal. Absorbance scales are equivalent. Spectra were normalized to 1824 cm\(^{-1}\). Spectra are labeled with time (in min) on the left hand side, with labels positioned relative to peak intensity at 3400 cm\(^{-1}\). Amide A, I, II, and III, associated with protein, are labeled for hard and gummy barnacles.
Following polymerization in air, a small amount of artificial seawater was added to the trough of the ATR element and polymerization was allowed to proceed in seawater for 3.5 – 4 hours (until no change in spectra was observed). Barnacles and seawater were removed from the ATR crystal after this time. In all trials, residual cement remained on the ATR crystal after the barnacles were removed, and could be seen as a thin opaque ring tracing the perimeter of where each barnacle had been. The opaque ring was uneven in appearance, and was more prominent for some barnacles than for others. Spectra of residual cement were taken until no changes in spectra were observed.

Residual cement spectra from both hard and gummy barnacles are shown in figure 41. Peaks are generally similar in position between hard and gummy residual spectra, though notable differences do occur. Several major peaks occur for hard residual within the 3079 – 2853 cm\(^{-1}\) region (likely –CH\(_3\) and –CH\(_2\) stretching vibrations) that do not appear for gummy residual. In hard residual cement, a minor peak occurs at 1736 cm\(^{-1}\), which does not appear in gummy residual cement. The –COOH stretch of protonated carboxyl groups occurs in this region (Barth and Zscherp 2002). Within the amide region, a small but consistent difference in amide I peak positions exist between hard and gummy barnacles; the amide I in hard residual is centered at 1639 - 1640 cm\(^{-1}\) whereas this peak in gummy appears at 1643 - 1646 cm\(^{-1}\). Amide I peak position is often indicative of protein secondary structure (Barth and Zscherp 2002; Jackson and Mantsch 1995). Difference spectra indicate that gummy residual is less ordered (more random coil configuration) than residual cement from hard barnacles.
Figure 41: FTIR of residual barnacle cement left by hard and gummy barnacles reattached to an ATR crystal. Data are from same barnacles shown in figures 39 & 40. Barnacles were reattached to an ATR crystal first in air until no change in spectra was observed (up to 23 hrs), then in seawater until no change in spectra was observed (up to 4 hrs). Spectra are normalized to 3700 cm$^{-1}$. Significant peaks are labeled in cm$^{-1}$. 
4.3.2.6 Trypsin Immunoreactivity

As described in Chapter 3, barnacle cement shows immunoreactivity toward full length bovine pancreatic trypsin antibody. Dot blotting was used to compare the intensity of trypsin immunoreactivity between cement collected in an unpolymerized state from hard and gummy barnacles. Immunostaining for trypsin antibody appeared at a greater intensity for unpolymerized cement from gummy barnacles than for unpolymerized cement from hard barnacles (Figure 42). This trend was consistent for all immunostaining conducted (a total of 21 dot blots for hard cement and 21 dot blots for gummy cement). Negative control dot blots (BSA and fibrinogen) did not stain. Differences in the appearance of cement dot blots (A vs. B shown in figure 42) are likely due to differences in hydration of the membrane when cement droplets were applied. Note that cement dot blots incubated in secondary antibody only appeared to stain immediately after antibody detection, but color quickly faded as the membrane dried. Staining did not fade for cement dot blots incubated with both primary and secondary antibody.
4.3.2.7 Trypsin Activity

Trypsin activity did not differ significantly between hard and gummy barnacles, as shown by trypsin substrate assays with BAPNA (Mann Whitney U rank sum test: \( p = 0.672 \)). Trypsin activity was \( 1.56 \times 10^{-6} \) BAPNA units (SEM ± 2.0 \( \times 10^{-7} \)) for hard barnacles and \( 1.72 \times 10^{-6} \) BAPNA units (SEM ± 3.1 \( \times 10^{-7} \)) for gummy barnacles (Figure 43). Note: 1 BAPNA unit is approximately 9000 BAEE units.
Figure 43: Mean trypsin activity (± SE) for hard and gummy barnacles using BAPNA (Nα-Benzoyl-DL-arginine 4-nitroanilide) as a substrate. Trypsin assays were conducted at pH 8.0. Samples were incubated for 1 hr at 37°C. Activity is shown in BAPNA units μl⁻¹ unpolymerized cement. n = 10 barnacles for both hard and gummy.

4.3.2.8 Transglutaminase Activity

Transglutaminase activity was shown for all individuals assayed, from both hard and gummy barnacles. Unpolymerized cement from gummy barnacles showed a greater range of transglutaminase OD₄₅₀ values (0.146 – 0.523; mean = 0.303, SEM ± 0.0343) as compared to hard barnacles (0.200 – 0.301; mean = 0.252, SEM ± 0.011). OD₄₅₀ values were not statistically different between hard and gummy barnacles (Figure 44; t-test: p = 0.237). Mean OD₄₅₀ for blanks was 0.1271 (SEM ± 0.004) and positive control (2 milliunits ml⁻¹ transglutaminase) was 0.449 (SEM ± 0.053).
Figure 44: Mean transglutaminase activity (± SE) for hard (n = 8) and gummy barnacles (n = 11). Assay was conducted using a cadaverine coated 96-well plate. Mean and SEM for blank (no enzyme present) and positive control are shown for comparison.
4.3.3 Release of Molecules from Silicone Substrates

Coupled gas chromatography-mass spectrometry was used to assess the presence and identity of molecules released from silicone foul release surfaces that had been used as barnacle growth substrates for 2½ years. With a 30 second methanol extraction, cyclic silicone monomers were identified for both T2® and Veridian® (Figures 45, 46 & 47). For Silastic T2® silicone, 7 major GC peaks were identified with mass spectrometry (Figure 45). 4 major GC peaks were identified with mass spectrometry trace for Veridian® (Figure 46). With the exception of dimethyl fluromethyl phenylsilane, all molecules that could be identified from Veridian® extractions were also present in T2® extractions.
Figure 45: GC Trace with mass spectrometry identification for Dow Corning Silastic T2® silicone, extracted with methanol. Name and structure (if available) of extracted molecules identified in the NIST database are noted.
Figure 46: GC Trace with mass spectrometry identification for International Paints Veridian® silicone, extracted with methanol. Name and structure (if available) of extracted molecules identified in the NIST database are noted.
Figure 47: Mass spec traces for GC peaks shown in figures 45 & 46 (red) with matching NIST mass spectral library traces (green).
4.4 Discussion

Genetic variability in the morphology of polymerized barnacle cement has been documented (Holm et al. 2005); when grown on silicone foul-release substrates, a portion of barnacles produce a thick, gummy cement as opposed to the typical thin, hard cement. Differences among individuals in cement morphology may result from genetic differences in the cement polymerization process. Biomechanical testing indicated that base plate composite modulus was significantly lower for gummy barnacles than for hard barnacles, but gummy barnacles tended to have a thicker base plate and therefore base plate flexural rigidity did not differ between hard and gummy barnacles. The data presented this study provide evidence that underlying biochemical differences exist between the cement from hard and gummy barnacles, in terms of protein content, relative abundance of individual protein components, polymerization time course, and chemistry of the polymerized cement. Furthermore, it was shown that molecules are released from silicone substrates and will be in direct contact with cement enzymes and structural components as they are released by barnacles.

4.4.1 Biomechanical Properties of Hard and Gummy Barnacles

Biomechanical testing of barnacle base plates was conducted in order to determine if differences in adhesive strength between hard and gummy barnacles can be solely attributed to differences in adhesive chemistry between these phenotypes or if differences in base plate mechanics (flexing) are also involved. A punch test apparatus was used to determine the base plate compliance of hard and gummy barnacles, from which composite modulus and base plate flexural rigidity was calculated. Modulus differed significantly between hard and gummy barnacle base plates, with hard barnacles showing a higher modulus than gummy barnacles. Mean modulus for hard
barnacles was more than twice that of gummy barnacles (6.5 GPa, SEM ± 0.6 for hard; 2.9 GPa, SEM ± 0.7 for gummy) when cement was not exposed to air prior to mechanical testing (the 14 mo. data). Lower modulus values for gummy base plates are likely due to a combination of incomplete curing of the cement (resulting in greater water content, as shown by water content assessment) and reduced mineralization of the cement. Incorporation of CaCO₃ into cement was originally suggested by Otness and Medcalf (1972) and later supported by Berglin and Gatenholm (2003). Crystallization of CaCO₃ would require a supersaturated solution for crystallites to grow (Dove and Hochella 1993) and therefore the hydrated gummy cement may not permit crystal growth. The pattern of lower modulus for gummy barnacle base plates is consistent with Sun et al. (2004) who used near-surface mechanical response by atomic force microscopy (AFM) to compare hard and gummy barnacle cement moduli. Whereas Sun et al. (2004) assessed only the near surface, proteinaceous cement regions, measurement in the current study included both the proteinaceous cement and the CaCO₃ base plate, resulting in 2-3 orders of magnitude higher moduli than those of Sun et al. (2004).

Despite the lower modulus of gummy barnacle base plates, the flexural rigidity of gummy barnacle base plates was not statistically different from hard barnacles. Gummy barnacles tended to have a thicker base plate than hard barnacles and this difference in thickness offset reduced base plate modulus, resulting in statistically similar flexural rigidity for hard and gummy barnacles. Wendt et al. (2006) and Holm et al. (2005) have shown that the force required to remove a gummy barnacle from a silicone substrate is less than the force required to remove a hard barnacle (with Holm et al. 2005 showing this trend in 1 of 2 silicones tested). As flexural rigidity did not differ between hard and gummy barnacles, mechanical differences in plate mechanics between hard and gummy barnacles are not likely to contribute to reduced adhesive strength in gummy barnacles.
Therefore differences in adhesive chemistry between gummy and hard barnacles are the primary factor resulting in differences in adhesive strength between these phenotypes.

4.4.2 Biochemical Properties of Hard and Gummy Barnacles

Genetic effects play a role in determining barnacle cement morphology when barnacles are grown on silicone foul-release substrates (Holm et al. 2005). This genetic variation is manifested either in terms of a physical mechanism, in which individuals respond to reduced adhesive strength on silicone in a heritable manner, or in terms of a chemical mechanism in which the chemistry of the cement produced by gummy barnacles differs from that of hard barnacles. The data presented in this study provide evidence that underlying biochemical differences exist between the cement from hard and gummy barnacles.

Prior to biochemical studies on hard versus gummy barnacles, it was predicted that gummy barnacles lacked a specific protein, set of proteins, or did not possess the total protein content necessary for complete polymerization of cement. This prediction was based on: 1) studies of defective human blood coagulation (reviewed in Lawn and Vehar 1986) in which deficiency of a single component of the blood coagulation cascade can lead to an inability to clot blood; and 2) studies with crustacean blood coagulation, in which hemolymph protein levels have been shown to be significantly lower in animals that are not able to clot blood than individuals who are able to clot (Jussila et al. 2001). This prediction, however, did not come to fruition. In fact, the concentration of protein tended to be higher in unpolymerized cement from gummy barnacles than from hard barnacles. Coomassie protein assays showed a trend toward higher protein concentration in gummy barnacle cement (significant at $p = 0.069$); SDS-PAGE of unpolymerized cement supported this trend, with cement from gummy barnacles consistently showing more intense protein bands than cement from hard barnacles.
(especially within the 150 – 75 kDa region). Just as too little protein content will inhibit protein coagulation, too much protein may also prevent coagulation from occurring in an optimal fashion. Assessing the affect of varying fibrinogen concentration on fibrin formation, Marx (2006) showed that extended clotting times occurred at both low and high fibrinogen concentrations when enzyme concentration was fixed. Clotting time was minimized between 1-10 μM fibrinogen, but was extended both above and below this optimum.

Barnacle families with a higher propensity to develop gummy cement showed more intense protein bands on SDS-PAGE. This observation is consistent with cement from gummy barnacles containing more protein than cement from hard barnacles. For the families that had either a high proportion of individuals with gummy cement on both silicone surfaces or a low proportion of individuals with gummy cement on both surfaces, protein band intensity (in the 150 – 35 kDa region) was similar for hard and gummy barnacles. This suggests that additional components (enzymes and/or co-factors) are necessary for expression of gummy cement; high or low protein concentration alone does not lead to gummy cement production.

At the resolution of SDS-PAGE, hard and gummy barnacles show a similar overall mix of protein bands; gummy barnacles did not consistently lack or possess a specific protein band. Bands on SDS-PAGE, however, are composed of many whole and cleaved proteins as shown in Chapter 2, and therefore differences in the presence or absence of specific proteins (if they exist) would likely not be resolved. Two-dimensional gel electrophoresis or another method of protein separation with greater resolution than SDS-PAGE may reveal specific proteins that differ between hard and gummy barnacles.

Unpolymerized cement from gummy barnacles tended to polymerize at a more rapid rate than did unpolymerized cement from hard barnacles, as shown by SDS-
Evidence of polymerization was observed in cement from gummy barnacles by 2 min and in cement from hard barnacles by 15 min. The time course of cement polymerization is consistent with Saroyan et al. (1970) who observed polymerization of barnacle cement within 15 min. This time frame is also consistent with clotting times shown for crustacean blood (within 2 min: Jussila et al. 2001) and human blood (4-15 min for whole blood: reviewed in Mann 1999; < 100 sec for isolated fibrinogen at optimal concentration: Marx 2006). As compared to cement from hard barnacles, the rapid polymerization and high protein content of cement from gummy barnacles results in an end-product cement that is less ordered and less well absorbed to the surface, as shown by FTIR (based on growth of amide regions over time and position of the amide I peak following polymerization: Barth and Zscherp 2002; Jackson and Mantsch 1995). This disordered, end-product cement is able to take up large amounts of water (Wiegemann and Watermann 2003; Wiegemann and Watermann 2004; Figure 34) and has reduced adhesive capability as compared to the cement of a hard barnacle (as described in the biomechanics section above).

The cause of an increased rate of protein polymerization in cement from gummy barnacles is likely due to the abundance of enzymatic components and/or cofactors contained in unpolymerized cement. Both trypsin activity and transglutaminase activity play a key role in barnacle cement polymerization (Chapter 3). Neither of these enzymes, however, occurred at a statistically higher concentration in gummy than in hard unpolymerized cement. Polymerization rate is likely dependent on a combination of both trypsin and transglutaminase concentrations. When in combination a minor, non-statistically significant difference in both trypsin activity and transglutaminase activity may result in the observed increased rate of polymerization in gummy cement. The concentration of cofactors, such as Ca$^{2+}$, which is required for both trypsin (Davie and
Fujikawa 1975) and transglutaminase activity (Sritunyalucksana and Soderhall 2000) may also play a key role in determining the rate of cement polymerization.

It is noteworthy that despite a statistically similar level of trypsin activity between unpolymerized cement from hard and gummy barnacles, immunostaining against bovine pancreatic trypsin antibody was consistently more intense for cement from gummy barnacles than for cement from hard barnacles. The discrepancy between activity assays and immunostaining provides evidence that other proteins, which show trypsin activity but are not immunoreactive toward bovine pancreatic trypsin, are present in barnacle cement and may be more abundant in hard than gummy barnacles. Subtle differences in enzyme components, and the substrate and co-factor specificity of these enzymes, may result in observed differences in polymerization chemistry between hard and gummy barnacles.

4.4.3 Release of Molecules from Silicone Substrates

Silicone foul-release substrates are clearly not inert. A 30 sec methanol extraction of silicone that had been used as a barnacle growth substrate for 2 ½ years resulted in seven identified gas chromatography peaks for T2® silicone and four identified GC peaks for Veridian®. Most identified molecules were cyclic siloxanes, although a silicone containing estrogenic compound was identified for both silicones and a fluorinated phenylsilane was identified for Veridian®. Other peaks were shown in the GC trace for both silicones that could not be identified, suggesting additional molecules were also released. The fact that leachable molecules were still present in silicone after 2 ½ years of use as a barnacle growth substrate (including initial presoaking in running seawater for 5 or 35 days, for T2® and Veridian® respectively) is significant and suggests that leaching of molecules from silicone is a long-term occurrence.
The concept that molecules are actually released from silicone foul-release substrates has been suggested previously by Meyer et al. (2006) and Berglin and Gatenholm (1999, 2003). Meyer et al. (2006) used contact angle measurements of a variety of liquids that mimicked components of marine organism adhesives to compare silicone substrates with and without added silicone oil. Contact angle anomalies (contact angles deviating from the trend expected with surface tension) were observed for coatings with added silicone oil both prior to, and following submersion (seawater and fresh water), suggesting long term elution of molecules from the silicone surface. Berglin and Gatenholm (1999, 2003) analyzed the cement of barnacles grown on silicone (PDMS: polydimethylsiloxane) and long-chain hydrocarbon (PMMA: polymethylmethacrylate) substrates using electron spectroscopy chemical analysis (ECSA). Silicone was found incorporated into the cement of barnacles grown on PDMS but was not found in the cement of barnacles grown on PMMA, indicating that uncross-linked silicone monomers are released from the silicone coating and incorporated into the cement.

This study not only supports the conclusions of Meyer et al. (2006) and Berglin and Gatenholm (1999, 2003) that molecules are released from silicone substrates over the course of several years, but also provides the specific identity of molecules released from commercial foul-release substrates. Knowing the identity of molecules released from silicone will allow testing of specific molecules or types of molecules to determine if these molecules have the potential to inhibit barnacle cement polymerization. Molecules released by the silicone substrate will be in direct contact with unpolymerized cement as it is released by the barnacle and as it polymerizes. The presence of these molecules may disrupt enzymatic activity and/or arrangement of cement structural components, at least in some (gummy) barnacles. Cement polymerization inhibition assays with anticoagulants (Chapter 3, gel electrophoresis) showed a greater difference in the
intensity of protein bands between anticoagulant treatments and control for gummy barnacles than for hard barnacles. The cement enzymatic and structural components of barnacles expressing the gummy cement phenotype may inherently be more sensitive to perturbation by inhibitors than are those of hard barnacles. Cement from gummy barnacles in isolation from inhibitors (biochemical assays above) show an increased rate of polymerization and an end-product cement that is less ordered than that of hard barnacles. Therefore the addition of molecules released from silicone to an already unstable cement results in an incompletely cured cement for gummy barnacles.

4.4.4 Conclusions: Production of Gummy Cement

Barnacle cement is a complex, multicomponent system (Kamino 2006) as is its polymerization (as shown in this document). As such, production of an incompletely cured, gummy cement is not likely to be caused by a single factor. The chemistry and adhesive interactions of hard and gummy barnacles clearly differ. This was shown by biomechanical analyses, which ruled out mechanical differences between hard and gummy barnacles as a factor contributing to reduced adhesive strength in gummy barnacles, and differing FTIR spectra of end-product cement from hard and gummy barnacles. Biochemical assays indicate that increased protein content along with some other factor (likely an enzyme or cofactor) results in polymerization that occurs at a more rapid rate in gummy than in hard barnacles. Rapid polymerization of a more protein-rich cement results in a disordered end-product cement with reduced adhesive capacity. On inert, high surface energy surfaces, differences in cement chemistry between hard and gummy barnacles are not expressed. On low surface energy silicone surfaces that release molecules from the surface, the already unstable, rapidly polymerizing cement of gummy barnacles is essentially ‘pushed over the brink’ by interaction with
released silicone molecules and/or reduction in the ability of contact dependent enzymes to operate.

The contribution of a physical mechanism to the production of gummy cement should not be ignored. As suggested in Chapter 1, the release of cement by barnacles is thought to occur through a ‘controlled bleeding’ mechanism. Physiological changes associated with molting allow for a small gap to form between the base and parietal plates, and upon muscular contraction hemolymph is forced through cement ducts into the gap. Gummy barnacles may inherently be ‘leakier’ than hard barnacles, with the size of the gap between the base and parietal plates or muscular control of the cement release process differing among individuals. Release of excess cement, that differs in chemical properties from that of hard barnacles may lead to a build-up of incompletely cured cement and result in the typical cupping of gummy barnacle base plates.
5. Conclusions

In this dissertation I have used evolutionary concepts to elucidate a complex biological phenomenon: the polymerization of barnacle cement. Specific evolutionary concepts include the tendency for proteins and biochemical mechanisms that work well to be conserved over time and the concept of evolution as a gradual process involving minor modifications over time. I have used well-studied biological phenomena, vertebrates and invertebrate blood coagulation, as a model in which to study barnacle cement polymerization. These systems are under similar selective pressures to barnacle cement polymerization and show similar chemical characteristics; blood coagulation and barnacle cement polymerization are both life or death situations in which soluble protein must quickly and efficiently coagulate. A brief summary of the key findings for each chapter, an integrated model for barnacle cement polymerization, as well as the significance of this work are presented in the following sections.

5.1 Chapter 2: Barnacle Hemolymph Functions as Cement

Acquisition of barnacle cement in the unpolymerized state is essential for the study of cement polymerization as well as full cement compositional analysis. A novel method for the collection of unpolymerized cement was described in this chapter. Multiple independent techniques (AFM, FTIR, chemical staining for peroxidase, tandem mass spectrometry) were used to verify that the collected secretion was cement. The identification of a large number of proteins besides ‘barnacle cement proteins’ with mass spectrometry, along with observations of hemocytes in unpolymerized cement inspired the hypothesis that barnacle cement is hemolymph. This hypothesis is supported by several lines of evidence including the ability for barnacle hemolymph to successfully clot (Fitzgerald 1968, Waite and Walker 1988, Kamiya et al. 2002). I suggest that the cement glands of the barnacle are associated with the circulatory system, and that cement ducts
facilitate controlled release of hemolymph at the periphery of the base plate. Upon release, hemolymph containing plasma (clottable protein) and cellular (enzymatic) factors clots when in contact with a foreign substrate. An immune response appropriate to a circulatory system that is temporarily open to the external environment is provided by plasma (lectins) and cellular (hyaline cells capable of phagocytosis) factors and potentially by peroxidase activity.

5.2 Chapter 3: Barnacle Cement Polymerization is Biochemically Similar to Blood Coagulation

Polymerization of barnacle cement bears a striking biochemical resemblance to vertebrate blood coagulation, both in terms of the polymerized protein aggregate and the polymerization mechanism. Similar to clotted fibrin, polymerized barnacle cement was shown to exist as a tightly interlocking mesh of fibrous protein, although the fibrous protein composing barnacle cement was biochemically distinct from vertebrate fibrinogen. Inhibitors of vertebrate blood coagulation, including heparin, trypsin inhibitor and Ca\(^{2+}\) chelators, impeded the polymerization of barnacle cement, suggesting that trypsin and Ca\(^{2+}\) are involved in the polymerization of cement. The presence and activity of a trypsin-like serine protease in barnacle cement was verified with multiple techniques, and was shown to be homologous to bovine pancreatic trypsin. Multiple trypsin-like proteases are likely present in cement and function in the activation of cement structural precursors, allowing for loose assembly with other structural proteins and rearrangement with the surface. Tandem mass spectrometry revealed a homologous protein to the catalytic subunit of human coagulation factor XIII (fibrin stabilizing factor: a transglutaminase that catalyzes covalent cross-linking between fibrin monomers) and amazingly, Western blotting showed this protein to occur at nearly the same molecular weight in barnacle cement as in human plasma. Transglutaminase activity was verified and likely functions to covalently cross-link loosely assembled cement monomers.
5.3 Chapter 4: Heritable Defects in the Coagulation Mechanism

Protein polymerization systems are inherently imperfect. Genetic variability in the morphology of polymerized barnacle cement has been documented; when grown on silicone foul-release substrates, a portion of barnacles produce a thick, gummy cement as opposed to the typical thin, hard cement. Altered cement morphology may stem from specific differences in the cement polymerization process, similar to those observed in other protein coagulation systems. Barnacles with gummy cement generally show reduced adhesive strength as compared to barnacles with hard, thin cement. Biomechanical testing indicated that unequal adhesive strength is due to differing adhesive interactions with the substrate between hard and gummy barnacles rather than different base plate mechanical properties. As a complex multicomponent system, it is unlikely that a single factor results in the formation of gummy cement. The data presented in this chapter provide evidence that underlying differences in cement chemistry occur between hard and gummy barnacles. Specifically, cement from gummy barnacles tended to have higher protein content than did cement from hard barnacles. Increased protein content, along with some other factor (likely an enzyme or cofactor) resulted in polymerization that occurred at a more rapid rate in gummy barnacles. The rapid polymerization of a more protein-rich cement led to a disordered end-product cement (as shown by FTIR) with reduced adhesive capacity in gummy barnacles. When barnacles are grown on low surface energy silicone substrates, the already unstable, rapidly polymerizing cement of gummy barnacles is essentially ‘pushed over the brink’ by interaction with silicone monomers (which were shown to be released from silicone surfaces) and/or reduction in the ability of contact dependent enzymes to operate, resulting in a gummy, improperly cured end-product cement.
5.4 Integrated Model for Barnacle Cement Polymerization

An integrated model for the polymerization of barnacle cement based on the data presented in this dissertation is shown in Figure 48. Hemolymph containing plasma and cellular components is released upon molting or injury. In combination, plasma and cellular components mediate both an immune response (shown in grey) and clotting. Clotting is dependent on structural components (in plasma), trypsin (potentially plasma or cellular) and transglutaminase (Factor XIII; cellular), all of which are likely released in an inactive form and subsequently activated. Both trypsin and transglutaminase activity is calcium dependent. Interaction of structural and enzymatic components results in a covalently cross-linked end-product cement. Trypsin activity associated with clotting results in the release of peptide pheromones, mediating a variety of behaviors in the marine environment including barnacle cyprid settlement induction and predator (gastropod) attraction (reviewed in Rittschof and Cohen 2004). High plasma protein concentration combined with sub-optimal enzyme and/or cofactor concentrations, and sub-optimal physical/muscular parameters (associated with the release of hemolymph) results in a gummy, improperly cured cement when polymerization occurs in contact with a low surface energy silicone coating and its associated leached molecules.
Figure 48: Schematic of barnacle cement polymerization as described in this dissertation. Black arrows indicate release, grey immune response, red enzymatic activity, blue regulatory activity, green release of peptide pheromones, and orange production of alternative (gummy) cement morphology.
5.5 Significance

The research presented in this dissertation, and the barnacle cement polymerization model based on that research, is broadly significant in terms of:

1) Management of biological fouling. The fouling of ship hulls costs the defense and shipping industries billions of dollars every year. Biological fouling has societal relevance in terms of reducing environmental degradation, energy usage (and therefore CO₂ emissions) and transport of introduced species. This research suggests specific molecules and classes of molecules that reduce adhesive strength of barnacles through the inhibition of cement polymerization.

2) Underwater adhesives and medical applications. The polymerization model presented in this dissertation will guide the creation of biomimetic adhesives that cure underwater or in vitro, by describing specific structural and enzymatic components necessary for adhesive polymerization.

3) Perhaps most importantly, this research provides clear examples of highly evolutionarily conserved proteins and biochemical mechanisms and demonstrates the power of applying evolutionary concepts in addressing complex biological phenomenon.
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