5-2008

DEVELOPMENT OF A NOVEL FOULING DETERRENCE STRATEGY BY UNDERSTANDING THE EFFECT OF NORADRENALINE ON THE CELLS OF EASTERN OYSTER, *Crassostrea virginica* AND CYPRIS LARVE OF THE STRIPED BARNACLE *Balanus amphitrite*

Neeraj Gohad  
*Clemson University*, ngohad@clemson.edu

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DEVELOPMENT OF A NOVEL FOULING DETERRENCE STRATEGY BY UNDERSTANDING THE EFFECT OF NORADRENALINE ON THE CELLS OF EASTERN OYSTER, Crassostrea virginica AND CYPRIS LARVE OF THE STRIPED BARNACLE, Balanus amphitrite

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

by
Neeraj Vijay Gohad
May 2008

Accepted by:
Dr. Andrew S Mount, Committee Chair
Dr. Joan Hudson
Dr. Thomas Boland
Dr. Andrew T. Metters
ABSTRACT

The focus of this study was to understand and evaluate the effect of noradrenaline (NA) on two prominent fouling marine invertebrates, the Eastern Oyster, *Crassostrea virginica* and the Striped Barnacle, *Balanus amphitrite* for the purpose of developing a novel fouling deterrence strategy.

To understand the effect of NA at a cellular level the immune cells (hemocytes) of *C. virginica* were chosen as representative molluscan cells. Upon stimulation with 10 μM of noradrenaline ~4% hemocytes of *C. virginica* labeled positive for the β-adrenergic receptor (β-AR). Upon NA stimulation the β-AR positive cells formed cell-cell synapses and apoptosis was detected in ~50% of the hemocyte population.

NA induced apoptosis was followed from the earliest onset to the terminal stages, from the release of cytochrome-c to the DNA degradation. Electric cell substrate impedance measurements suggested that NA stimulation induced cytoskeletal changes in the hemocytes. SDS PAGE and Western analysis corroborated the presence of β-AR on NA stimulated hemocytes. Mass spectral analysis of the receptor protein revealed that the putative hemocyte β-AR has a 30% sequence identity to the human β-AR.

Effect of NA on the settling behavior of *B. amphitrite* cypris larvae was evaluated by challenging the larvae with micromolar concentrations of NA ranging from 30-100 μM. The noted searching behavior of cypris larvae was lost after NA challenge and the larvae failed to cement to the substratum. ~70% of the NA treated larvae failed to settle and cement. NA treatment caused a considerable delay in cyprid-adult metamorphosis.
Metamorphosed juvenile barnacles were observed 96 hours following NA treatment, whereas the control cyprids metamorphosed within 24-48 hours. In the cyprids-adult metamorphosis remnants of the cyprid stage were observed up to 48 hours of development. Metamorphosed juvenile barnacles appeared normal from the presence of growth increments and beating of cirri. NA challenge seemed to promote metamorphosis to juveniles forgoing the settling behavior and cementation to the substratum.

To assess the applicability of NA as a fouling deterrent, NA molecules were covalently conjugated to HEMA (2-hydroxyethyl methacrylate) and MAA (methacrylic acid) polymer surfaces. Ability of covalently conjugated NA molecules to activate their target adrenoreceptors was assessed by understanding the effect of NA-conjugated polymer surfaces on hemocytes of *C. virginica*.

NA conjugated polymer surfaces induced apoptosis in the hemocytes of *C. virginica*. Annexin-V assay confirmed the initiation of apoptosis.

Cytoskeletal structure of the hemocytes adhering to NA-conjugated polymer surfaces displayed pronounced degradation. Apoptotic blebbing of plasma membrane was also observed using scanning electron microscopy. Control polymer surfaces failed to exert any deleterious effects on the hemocytes.

Based on the results obtained in this study a novel fouling deterrence strategy is discussed.
DEDICATION

This dissertation is dedicated to my parents Meera and Vijay Gohad, their love, support and encouragement has enabled me to pursue my dreams.
ACKNOWLEDGMENTS

I am grateful to many people who have helped me in numerous ways towards the completion of my educational endeavor. Foremost, I would like to thank Dr. Andrew S. Mount, my major advisor who gave me many opportunities to grow not only as a scientist but also as an individual.

I would also like to thank my committee members, Drs. Andrew Metters, Joan Hudson and Thomas Boland. Their critical questions motivated me to evaluate my research from different perspectives. A special thanks to my dear brother, Nikhil Gohad, who always encouraged me throughout this journey. Very sincere thanks to my Guru Dr. Ashok Marathe, who has had positive influence on me. Thanks to my best friend Pranjal Nahar for keeping me cheerful through the ups and downs of research.

I would also like to thank my good friend and collaborator Nihar Shah, for a very productive collaboration. I am also grateful to Dr. Dale Layfield, who guided and helped me organize my poster presentations, his valuable aid made my presentations a success.

I would also like to thank my collaborators Dr. Dan Rittschof and Beatriz Orihueladiaz at the Duke University Marine Laboratory for providing cypris larvae for my research.
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CHAPTER ONE
INTRODUCTION

Biofouling is the undesirable accumulation of bacteria, algae, plants and marine animals on submerged structures such as ship hulls, oil rigs, dock pilings and other submerged structures in marine environments. Biofouling leads to aesthetic and structural disintegration and increased surface roughness (Olsen, Pedersen et al. 2007). In a marine environment biofouling initiates with the adsorption of dissolved macromolecules, such as free amino acids, proteins, peptides and carbohydrates, which takes place in a very short time from minutes to hours depending on the nature of the surface (Coughenower and Curl 1975; Amano, Hara et al. 1982; Wahl 1989; Yebra, Kiil et al. 2004). Macromolecular adsorption is succeeded by colonization of microfouling organisms such as bacteria, diatoms, protozoans, algal spores and other microorganisms (Wahl 1989; Yebra, Kiil et al. 2004). Microfouling may lead to macrofouling as many of the microfoulers serve as settlement cues for invertebrate larvae (Chalmer 1982; Yebra, Kiil et al. 2004). Dependent on the geography and the season, a macrofouling community may consists of soft foulers such as sponges, hydroids, ascidians and calcareous hard foulers such as tube-worms (polychaetes), barnacles, bivalve mollusks, bryozoans, tunicates, cnidarians, (Richmond and Seed 1991; Yebra, Kiil et al. 2004; Koehl 2007; Mitbavkar and Anil 2007).

Micro or Macro fouling leads to increased surface roughness of ships which translates into high frictional resistance leading to increase in weight, reduction in speed
and loss of maneuverability. To offset these effects fuel consumption is increased which in turn leads to harmful emissions.

Increase in fuel consumption can be up to 40% and the cost of the voyage can increase as much as 77% (Yebra, Kiil et al. 2004). Biofouling also leads to structural and aesthetic deterioration (also reviewed in chapter 3). Attachment mechanisms deployed by calcareous biofoulers have high adhesive strengths (Holm, Kavanagh et al. 2006) and can cause structural damage to the underlying surfaces. Calcareous biofouling can also be very difficult to remove, requiring specialized equipment and in the process can generate hazardous waste which requires proper treatment prior to disposal.

Biofouling has been combated for over 2000 years of maritime history (Yebra, Kiil et al. 2004). Early Phoenicians and Carthaginians used pitch and possibly copper sheathing on ship’s bottoms while wax, tar and asphaltum were used by other ancient cultures. Coatings of arsenic and sulfur mixed with oil were used to combat molluscan shipworms. In the 3rd century B.C., the Greeks used tar, wax and even lead sheathing. The Vikings in 10 A.D. used “seal tar” to coat their vessels. From the 13th to the 15th century, pitch was extensively used to protect ships, sometimes blended with oil, resin. Hide sheathing was another material used in the 14th century. In the early 16th century, wooden sheathing was put over a layer of animal hair. Prior to the 18th century lead sheathing was commonly used and was officially adopted by Spain, France and England. As lead caused corrosion of the iron components on the ships such as the rudder, it was abandoned by the British admiralty in 1682. Wooden sheathing painted with mixtures of tar, grease, pitch, sulfur and was studded with iron or copper nails. The nails were set so
closely to each other that they formed a metallic sheath. In 1863, James Tarr and Augustus Wonson received a US patent for an antifouling paint formulation containing copper oxide in tar with naphtha or benzene. By the end of the 19th century, ‘Italian Moravian’ and McInness ‘hot-plastic paints’, shellac type paints (rust preventive) and various copper bases paints were widely used. (Yebras, Kiil et al. 2004).

In 1958 Montermoso and co-workers first suggested the possibilities of using TBT (tri-butyl tin) acrylate esters as antifouling coatings. Six years later, James patented the use of organotin copolymers including copolymers of TBT acrylate and methyl methacrylate. This was followed by the invention of TBT self-polishing copolymer (TBT-SPC) technology by Milne and Hails in 1974 which revolutionized the antifouling paints and the shipping industries. Triorganotin derivatives were extensively used as they had wide-ranging biocidal effects and did not cause galvanic corrosion on aluminum hulls. The addition of bis-oxide TBTO and the fluoride TBTF to both insoluble and soluble matrix paints produced most potent antifouling coatings (Yebras, Kiil et al. 2004). Although TBT based coatings proved extremely efficient, they caused tremendous damage to the environment, including causing shell deformities in oysters and imposing male sex organs in female snails of the dog-whelk, nucella spp. In recognition of the devastating effects caused by TBT, an international convention held on 5 October 2001, required that the parties to the convention ban the application of TBT-based antifouling paints from 1 January 2003, and remove such paints from the surface of commercial and recreational vessels from 1 January 2008 (Yebras, Kiil et al. 2004).
In the post TBT era, two main strategies deployed against biofouling are 1) The traditional and widely used approach which involves the use of biocides incorporated into coatings. Amongst such coatings are the i) self-polishing coatings (SPC-paints) in which hydrolysis of covalent bonds leads to release of copper. ii) Soluble matrix paints copper in which copper is dissolved in water soluble binder, dissolution of binder releases copper. iii) Insoluble matrix paints (also termed contact leaching or continuous contact); the polymer matrix is insoluble and does not polish or erode after immersion in water. A variety of commercial high molecular weight polymers are used, such as the insoluble vinyl, epoxy, acrylic or chlorinated rubber polymers. 2) Antifouling coatings termed non stick or fouling release which depend entirely on surface characteristics to release fouling. Such coatings prevent biofouling by providing ultra-smooth, low friction surfaces on which organisms have difficulties in settling. Variations of fluoropolymers and silicones are used in commercial fouling release coatings (Yebra, Kiil et al. 2004). Although these surfaces get fouled, the fouling is released by the shear forces exerted when the vessel moves through the water. (Yebra, Kiil et al. 2004).

Preceding attachment and metamorphosis to juveniles, invertebrate larvae explore the substratum (Crisp 1974; Pawlik 1992; Hadfield 1998). Molluscan veliger and barnacle cypris larvae investigate surfaces using exploratory structures bearing an array of sensory cells (Clare 1995; Zimmer-Faust, Tamburri et al. 1996; Walters, Miron et al. 1999). The choice to attach and metamorphose is made upon receiving favorable cues.

The cyprid is the stage in the life cycle of cirripedes that selects a suitable substrate for settlement and completion of metamorphosis to adult barnacles (Nott and
Barnacle cyprid behavior prior to attachment and metamorphosis has been extensively documented (Kinght-jones 1953; Crisp 1961; Crisp 1962; Crisp and Meadows 1962; Crisp 1974; Khandeparker, Anil et al. 2002) and it is recognized that the cyprids are able to detect and respond to the physical nature of substrates as well as soluble and insoluble chemical cues present on or in the proximity of the substrates (Nott and Foster 1969; Clare and Nott 1994; Lagersson, Garm et al. 2003).

Cyprids investigate the substratum using a pair of antennules comprised of four segments (Visscher 1928; Crisp 1961; Lagersson and Hoeg 2002). Cyprids utilize their antennae in a staggered bipedal walking manner for exploration (Lagersson & Hoeg, 2002) during which the fourth antennular segment is flicked in a repeated manner. The flicking movement probably brings the setae of the forth segment in proximity or even in contact with the substratum, perhaps increasing the stimulus access to the setae (Clare and Nott 1994; Lagersson, Garm et al. 2003) also reviewed by Dahlström (2004). Cyprids appear to be partial to surface adsorbed molecules than to soluble stimuli (Crisp and Meadows 1962; Crisp and Meadows 1963). This ‘tactile chemical sense’ is defined by Crisp (1963) as the sense experienced by genuine contact with an adsorbed or insoluble material. The gregariousness of cyprids guarantees settling conditions favorable for adult life and cross fertilization (Nott and Foster 1969). Prior to attachment oyster veligers explore a substratum by means of their foot (Visscher 1928; Coon, Fitt et al. 1990). The Molluscan larval foot is a complex organ used for locomotion and attachment and surface exploration (Croll 2006). Croll et al (2006) have observed that foot of the pediveliger larvae of the Blue Muscle, *Mytilus edulis* (Mytilidae) and the deep sea scallop,
*Placopecten magellanicus* (Pectinidae) contains catecholaminergic cells, some of which possess processes that traverse the epithelium and bear long cilia projecting form the sole of the foot. These peripherally located somata are connected to the pedal ganglia via fiber pathways (Croll 2006).

Biogenic amines synthesized from decarboxylation of amino acid are classified into indoleamine (serotonin), catecholamines (dopamine, noradrenaline and adrenaline), monoamines (octopamine and tyramine), imidazole (histamine) and amino acid (γ-amino butyric acid (GABA)) (Roeder 2002) reviewed in (Dahlström 2004).

In marine invertebrates catecholamines induce neuroendocrine changes and appear to divert the organism’s energy resources away from physiological functions such as growth, reproduction and immunity. These changes induce metabolic and behavioral adaptations that may help the animal to overcome the threat to survival (Ottaviani and Franceschi. 1996; Maule and Vanderkooi. 1999; Lacoste, Jalabert et al. 2001). Noradrenaline (NA) is the principle catecholamine circulating in oysters (Lacoste, Malham et al. 2001), other biogenic amines influence a variety of physiological processes in mollusks, such as feeding (Teyke., Rosen. et al. 1993), locomotion (Sakharov and Sala`nski 1982), reproduction (Martínez and Rivera 1994) as well as larval settling and metamorphosis (Pires, Coon et al. 1997). Biogenic amines serotonin, octopamine, tyramine, dopamine and histamine are also known to be present in invertebrates (Dahlström 2004). Oyster larvae have NA and epinephrine (Coon and Bonar 1986) and barnacle cypris larvae possess dopamine and serotonin (Yamamoto, Katsuhiko Shimizu et al. 1999). Biogenic amines have been distinguished into three different classes
depending on the system they act on: i) classical neurotransmitters ii) neuromodulators iii) neurohormones. Neuromodulators affect a broader tissue area than the neurotransmitters as they bind to GPCRs and initiate an array of signal transduction cascades (Dahlström 2004).

Noradrenaline has been shown to induce metamorphosis without settlement in the larvae of the Eastern and the Pacific oysters (Coon, Bonar et al. 1986). Dopamine and serotonin agonists and antagonists have been shown to inhibit larval settlement in barnacle *B. amphitrite* (Yamamoto, Katsuhiko Shimizu et al. 1999). Adrenoreceptor agonists in solution and adsorbed on a surfaces have been shown to inhibit barnacle *Balanus improvisus* larval settlement (Dahlström, Mårtensson et al. 2000; Dahlström 2004; Dahlström, Lindgren et al. 2005). Adrenoreceptor antagonists have also been shown to inhibit larval settlement in *B. amphitrite* (Cirripedia), *Bugula neritina* (Bryozoa) and *Hydroides elegans* (Polychaeta). Thus the concept of interfering with the surface exploration and attachment mechanisms involved in larval settlement is being explored as the most promising and environmentally benign antifouling option (Burgess, Boyd et al. 2003; Yebra, Kiil et al. 2004).

The involvement of adrenoreceptor signaling in invertebrate larval settlement and metamorphosis pathways is well demonstrated by these studies. Majority of these studies have focused on screening such pharmacological agents to investigate their ability to inhibit larval settlement. Little information is thus available about the effects of adrenoreceptor agonists or antagonists at a cellular level, or effects of such compounds on the larval ecdysis and metamorphosis. As the neurotransmitters and neuromodulators are
potent even at extremely low concentrations from nano-moles to pico-moles, the use of such compounds as biocides deployed in leachable marine paints may not be permitted by various environmental protection agencies of the world. The mechanism through which such compounds manifest their effects on target species is yet to be fully understood and the effects these compounds may have on non-target species is an area still largely unexplored. Thus it may not be wise to deploy such agents in antifouling coatings if are to avoid the environmental devastation similar to that caused by TBT.

Thus the focus of this dissertation is to understand the cellular and signal transduction mechanisms mediated by adrenoreceptor agonist noradrenaline and the effect it has on the larval ecdysis and metamorphosis. A novel strategy of covalently conjugating adrenoreceptor agonists to polymer surface is also investigated. Such a strategy would eliminate the need of a biocide leaching out of the paint. For this study the catecholamine noradrenaline was chosen as a candidate adrenoreceptor agonist. Noradrenaline (NA) has been demonstrated to inhibit oyster larval settlement while inducing metamorphosis via $\alpha_1$-adrenoreceptor activation. Although NA prevented oyster larval settlement, it induced metamorphosis and was not toxic to the larvae. (Coon, Bonar et al. 1986; Coon and Bonar 1987). Effects of noradrenaline have been studied extensively by Lacoste et al from a molluscan immunological perspective. Noradrenaline has been shown to be the principle circulating catecholamine in the hemolymph of C. gigas and is up-regulated when oysters are under environmental stress (Lacoste, Malham et al. 2001). NA has also been shown to induce apoptosis in the hemocytes of C. gigas through the $\beta$-adrenergic receptor signal transduction pathway (Lacoste, Cueff et al.
2002). As adrenoreceptor agonists have been shown to inhibit larval settlement in *B. improvisus*, it was hypothesized that NA would also be able inhibit larval settlement in *B. amphitrite* while promoting metamorphosis.

The dissertation is comprised of three manuscripts constituting three chapters which are briefly introduced hereafter.

I. Catecholamine noradrenaline induces apoptosis in hemocytes of Eastern Oyster *Crassostrea virginica*: Its potential as a fouling deterrent.

NA has been shown to induce apoptosis in hemocytes of oyster *C.gigas* through the β-adrenergic receptor (Lacoste, Cueff et al. 2002) at near physiological concentrations. The effect of NA concentrations relevant to inhibit larval settlement was assessed on the hemocytes of Eastern oyster *C.virginica*. Hemocytes were chosen, as the system is amenable to experimental manipulation and assays developed could be applied for studying the effects of NA and other biogenic amines on invertebrate larvae. The goal of this study was also to identify the specific adrenergic receptor present on the oyster hemocytes which serves as the target receptor for NA. Approximately 4% of the oyster hemocytes possessed the β-adrenergic receptors which were up-regulated upon stimulation with 10 μM NA. Upon stimulation with NA, these 4% hemocytes mediated apoptosis in
approximately 50% of the hemocyte population. Induction of apoptosis was followed from its earliest onset to the terminal stages.

The receptor protein was isolated from NA stimulated hemocytes and identified by western blotting analysis using a nitr-β-adrenergic receptor antibody. The isolated receptor complex was further analyzed by mass spectrometry. The sequence obtained from mass spectrometry had a 30% homology to the human β-adrenergic receptor. β-adrenergic receptor positive cells were able to mediate an effect on the surrounding cell population upon NA-stimulation, through a mechanism which remains to be investigated. This indirect effect mediated by hemocytes could perhaps suggest the presence of a paracrine signaling mechanism in the oysters.

II. Noradrenaline inhibits settlement behavior and attachment in the cypris larvae of barnacle *Balanus amphitrite*

Noradrenaline has been shown to inhibit larval settlement behavior and attachment in oyster larvae, NA treated oyster larvae metamorphose without cementation to a substratum (Coon and Bonar 1987). α₁-adrenergic receptor agonist medetomidine and clonidine have been shown to inhibit cypris larval settlement in *B.improvisus* (Dahlström 2004). Majority of these investigations have focused on understanding the activity of potential antifouling compounds only on cyprid settlement. Thus little information is available on the effects of
these compounds on cyprid morphology and cyprid-barnacle metamorphosis. Concentrations of NA from 30 μM-70 μM inhibited the settlement behavior in the cypris larvae of *B. amphitrite*. Approximately 70% of NA treated cyprids did not settle. NA treated cyprids metamorphosed into juvenile barnacles without cementation to the substratum. Ecdysis was considerably delayed in NA treated barnacles when compared to untreated controls. Cyprid-barnacle metamorphosis was also delayed and some of the features of the cyprid stage such as the antennules were identifiable till the 48-72 hours post NA treatment. Although NA treatment delayed the cyprid-barnacle ecdysis, it did not manifest any toxic effects on the cypris larvae.

**III.** Noradrenaline molecules covalently conjugated to polymer surfaces induce apoptosis in hemocytes of Eastern Oyster *Crassostrea virginica*: A novel fouling deterrence strategy

Exploration of surfaces prior to settlement is a hallmark of marine invertebrate larvae. Barnacle cyprids and oyster pediveligers are able to detect surface immobilized chemical cues. Thus it is hypothesized that covalent conjugation of noradrenaline or analogous molecules to a polymer surface would eliminate the need of incorporating biocides in leachable coating systems. NA is an agonist for the adrenoreceptor which belongs to the G-protein coupled receptor family (GPCR). Covalently conjugated NA molecules attached to the polymer
surfaces cannot be internalized by cells. Thus it is essential to understand if such surface attached molecules could activate GPCRs of the adhering cells. NA molecules were covalently conjugated to polymer surfaces and hemocytes of the oyster *C. virginica* were allowed to interact with the surfaces. NA-conjugated polymer surfaces induced apoptosis in the hemocytes which was detected by translocation of phosphatidylserine to the outer leaflets of the plasma membrane. Cytoskeletal structure of the hemocytes adhering to NA-conjugated polymer surfaces deteriorated, possibly as a consequence of apoptosis or perhaps through incessant signaling and phosphorylation of cytoskeletal elements mediated by the activated GPCR. A novel fouling deterrence strategy is discussed.
Abstract

We report a class of noradrenaline (NA) responsive oyster hemocytes termed β-adrenergic receptor cells (β-AR cells). β-AR cells comprise only 4% of the total hemocytes population. NA induced apoptosis in the oyster hemocytes which was detected from its earliest onset to the terminal stages. Impedance studies revealed that the β-adrenergic receptor complex responds within a few seconds of stimulation, downstream cellular effects occur within minutes and the complex resets within 30 minutes. It appears that β-AR cells induce apoptosis in other hemocytes. Identity of the β-AR was confirmed by western blotting and mass spectral analysis. The putative β-AR was found to have 30.32% homology to the human β-AR.
Introduction

The Mollusca is one of the most successful animal phylum found in aquatic and marine environments. Sessile bivalves such as oysters, mussels and shipworms pose significant fouling problems for vessels and underwater structures. In aquatic environments, Zebra mussels, a fugitive species introduced to the Great Lakes in 1986, form large congregations which often fouls intake pipes of municipal water plants and commercial power utilities (Ludyanskiy, McDonald et al. 1993). In the marine environment, the shipworm, a marine wood boring bivalve, is a serious threat to piers, pilings and other submerged wooden structures (Uda, Dieter et al. 2002). To date, most of the antifouling biological research has focused on understanding the chemical ecology of natural antifouling defenses including the isolation of secondary metabolites with biocidal potential (Tegtmeyer and Rittschoff 1998; Pereira, Carvalho et al. 2002; Rittschof, Lai et al. 2003) and studies on the inhibition of larval settlement with emphasis on ascertaining bacterial cues and surface antifouling characteristics of marine biofilms (Bers and Martin 2004). Little is known about the mechanisms of cellular interaction of these molecules with molluscan larvae, even less when molecules are immobilized as a component of an antifouling polymeric coating.

The β-adrenergic receptors (β-AR) have been well described in adult oysters in the pioneering work of Lacoste et al from an immunological and neuroendocrinological standpoint (Lacoste, Malham et al. 2001; Lacoste, Malham et al. 2001). Studies on the effects of catecholamines, ligands for the β-adrenergic receptors, have also been carried
out on larvae of pacific oyster *Crassostrea gigas*. These catecholamine treated oyster larvae lose their ability to settle and cement to a substratum and metamorphose into normal juvenile oysters. A process extensively studied by oyster hatcheries for cultchless production of oysters (Coon, Bonar et al. 1986; Coon and Bonar 1987). Inhibition of settlement and cementation of a hard fouling organism such as the oyster by noradrenaline is an important phenomenon, understanding of which will shed new light on marine invertebrate biofouling. In this study we investigate and discuss the effect of NA on oyster hemocytes and its potential application as a fouling deterrent.

Due to difficulty in accessibility, it is very not easy to ascertain the effects of noradrenaline on larval cells. Hence in the current study we focus on understanding the effects of noradrenaline (NA) on the hemocytes of adult eastern oyster, *Crassostrea virginica*. Various cell and molecular biology assays developed in this study will be used in successive studies to understand effects on NA on oyster larval cells. NA is a known ligand for the β-adrenergic receptors. Role of β-adrenergic receptors in inducing apoptosis upon binding to its agonists has been well studied (Ma and Huang 2002). We hypothesized that NA could serve as potent fouling deterrent by inducing a stress response in molluscan hemocytes. To test this hypothesis focus will be given on understanding the apoptotic cascade mediated by NA in oyster hemocytes.
Materials and methods

Oyster Culture

Eastern oyster adults *Crassostrea virginica* were purchased from Pemaquid Oyster Company Inc. (Waldoboro, Maine). After receiving, the oysters were held in a 180 gallon (681 liter) tank at 18°C in artificial sea water at 31% salinity with saturating levels of dissolved oxygen. The animals were fed twice a week with Shellfish Diet 1800® (Reed Mariculture Inc).

Hemocyte and NA preparation

The oyster’s shell was notched with a cement saw to enable hemolymph extraction from the adductor muscle. A 22 gauge needle affixed to a disposable syringe was inserted into the adductor muscle and approximately 1 ml of hemolymph was withdrawn (Lacoste, Cueff et al. 2002). All stimulations with NA and fixation of the cells was carried out at 18°C (Bigas, Durfort et al. 2006). Hemolymph osmolality was measured using the Advanced Instruments Micro-Osmometer. Noradrenaline (DL-Noradrenaline hydrochloride) (NA) was purchased from Sigma-Aldrich Corp (Cat#74490). A 10 μM solution of NA was prepared in molluscan ringers solution in which the osmolality was matched to the hemolymph (Singh 1938). This particular concentration for NA was chosen based on previous observations in hemocytes of pacific oyster *C. gigas* to NA stimulation, at slightly above physiological and elevated
concentrations of NA, immune function such as production of reactive oxygen species is stimulated (Lacoste, Malham et al. 2001). Hemocyte Phagocytosis is adversely affected (Lacoste, Malham et al. 2001). In hsp transfected hemocytes expression of hsp70 (heat shock protein, a homeostatic mechanism induced both in prokaryotes and eukaryotes) is transcriptionally up regulated. (Lacoste, De Cian et al. 2001). The concentration of NA at 10 μM was purposely maintained well above the physiological concentration to induce a stress response in hemocytes.

Detection of the β-adrenergic receptor complex in cells

Hemolymph, ~0.4 ml was added to 8 well chamber slides (Lab-Tek II chambered coverglass, Fisher cat# 12-565-470) and incubated for 1 hour at 18°C for allowing the cells to settle and adhere. Non adherent cells and serum were removed by gentle washing with molluscan Ringer’s solution. The remaining adherent cells were incubated for 20 minutes after 10 μM NA solution was added to the chamber slide. To remove NA, the cells were washed with molluscan Ringer’s solution and fixed with 4% paraformaldehyde in molluscan Phosphate Buffer Saline (PBS) (PBS was adjusted to match hemolymph osmolality). Cells were fixed for 30 minutes at 18°C. After fixation the cells were washed 3 times with molluscan PBS to remove any un-reacted paraformaldehyde. The hemocytes were blocked using 4% normal goat serum (NGS) for 1 hour. The cells were the incubated with the anti-β-adrenergic receptor primary antibody (Calbiochem, Cat #PC162) at a 1000 fold dilution for one hour. Secondary labeling was accomplished with
a fluorescent anti Alexa Fluor 488 rabbit-IgG antibody (Invitrogen Cat #A31623) at 2000 fold dilution for 1.5 hours. Between the blocking steps and incubations for both the antibodies, the cells were washed 3 times for 5 minutes each with molluscan PBS. Hemocytes in the control wells were stimulated with molluscan ringer’s solution lacking NA, the cells were labeled in the same manner as NA stimulated cells. To ascertain specificity of the fluorescent secondary antibody, hemocytes were stimulated with NA, fixed and blocked as described above and directly labeled with fluorescent secondary antibody, excluding labeling with primary anti-β-AR antibody. After labeling, the cells were analyzed with Zeiss LSM 510 confocal laser scanning microscope (Zeiss Corp, Thornwood, NY) with three different laser lines and appropriate filter sets.

Apoptosis studies

Three different assays were used to detect the onset and progression of programmed cell death in oyster cells, release of cytochrome-c from mitochondrial membranes, detection of a phosphatidylserine flip from the inner to outer cytoplasm membrane by fluorescent annexin-V and DNA fragmentation by APO-BrdU TUNEL assay. The cytochrome-c assay protocol was followed as provided in the kit (Invitrogen corp; catalog #S35115). NA stimulated hemocytes were fixed with paraformaldehyde as described previously. Hemocytes were permeabilized with 0.2% Triton X-100, blocked with 10% normal goat serum (NGS). The cytochrome-c was labeled with a 500 fold dilution of the anti-cytochrome-c antibody. The protocol was modified following
labeling with cytochrome-c antibody; cells were subsequently washed with molluscan PBS and blocked using 10% NGS, washed again and labeled for the β-AR using the anti-β-AR IgG. Following primary labeling, the anti-cytochrome-c IgG was secondarily labeled with Alexa Fluor 488 goat anti–mouse IgG. After this, the cells were washed in molluscan PBS and the β-AR was secondarily labeled with Alexa Fluor 594 goat anti–rabbit IgG. The cells were subsequently washed three more times with molluscan PBS. Control cells were incubated with molluscan PBS in the absence of NA and processed in the exact same manner as described above. Cells were visualized using the Zeiss 510 LSM.

To detect the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet, the outer leaflet was labeled with FITC labeled Annexin-V (Lacoste, Cueff et al. 2002; Sokolova, Evans et al. 2004). The Annexin-V assay protocol was followed as provided in the kit (Invitrogen, catalog # V13241). NA stimulated and paraformaldehyde fixed hemocytes were assayed. Following this cells were washed with molluscan PBS and labeled for β-AR as described previously. The cells were visualized using the Zeiss 510 confocal LSM.

For detecting the late stages of apoptosis induced by NA, an APO-BrdU TUNEL Assay (Invitrogen; catalog# A-23210) was performed. The assay was carried out based on the suggested protocol from the kit. NA stimulated and fixed cells were washed with molluscan PBS to remove unreacted paraformaldehyde. Cells were incubated with terminal deoxynucleotidyl transferase (TdT), which adds deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP), in a template-independent fashion, thus
labeling the break sites on the fragmented DNA. The BrdUTP incorporated into the DNA nicks was detected using Alexa Fluor 488 conjugated Anti-BrdU mouse monoclonal IgG. At this step the protocol was modified, the cells were washed in molluscan PBS, blocked using 10% NGS and labeled for β-AR as described previously. Cells were visualized by the Zeiss 510 LSM.

Cell count determination

For all the immunofluorescence assays, cell counts and percentages were determined in the following manner. For each experiment ~1-1.5 ml hemolymph was drawn from 4 different oysters and approximately ~0.4 ml of hemolymph was introduced in each well of the 8 well chambered coverglass, 2 such 8 well chambered coverglass were used for each experiment. Each experiment was repeated 3 times with above mentioned parameters. For determining cells counts, in each of the experiments (+) and (-) cells were counted in 10 different fields in each well of the 8 well chamber slides, mean counts were ascertained for each well and then mean counts were averaged for each chamber slide.
Electric cell substrate impedance sensing (ECIS)

ECIS studies were carried out using the ECIS Model 1600 system supplied by Applied Biophysics (Qiao, Huang et al. 2006). Eight well electrode arrays (Applied Biophysics; catalog# 8W10E) were washed and allowed to equilibrate with the molluscan Ringer’s solution. Hemolymph was extracted as described earlier. The molluscan Ringer’s was decanted from the electrode arrays and the hemocytes were allowed to adhere and settle for 1 hour. Basal impedance readings were taken at 15 kHz as hemocytes settled and adhered to the surface of the array. The hemocytes were stimulated by adding 10 μM NA solution while the impedance was recorded and logged. Molluscan Ringer’s solution was added to a control well of hemocytes at the same time as the treatment and the impedance was recorded and logged.

SDS-PAGE, Western blotting and proteomic analysis

Hemolymph was extracted as described previously. The cells were incubated with 10 μM NA for 20 minutes. Following incubation, cells were lysed with 1% SDS in de-ionized water followed by sonication using a Thermo-Fisher Model 100 sonic dismembrator. Protein extractions were accomplished according to the method outlined in (Rapp, Munson et al. 1986). Total protein was estimated by BCA assay (Pierce Chemical; catalog# 23225). SDS-PAGE was performed using NuPAGE 4-12% Bis-Tris Gels (Invitrogen; catalog# NP0321) and ran in XCell SureLock Mini-Cell (Invitrogen;
Electrophoresis was performed at 120 volts for 1 hour. The gels were stained with Simply Blue Safe Stain (Invitrogen; catalog# LC6060).

To improve the detection of receptor proteins, a protocol was followed which can visualize proteins present in concentrations of less than 25 nanograms (Rapp, Munson et al. 1986). Prior to western transfer, gels were zinc stained using the Zinc Stain and Destain Kit (Bio-Rad; catalog# 161-0440) in order to check the efficiency of electrophoresis before proceeding to Western transfers (Wong, Sridhara et al. 2000). Western transfers were carried out using Criterion Blotter (Bio-Rad; catalog# 70-4071) on PVDF (Polyvinilidyn difluoride) membranes. Western blotting was performed using a WesternBreeze™ chromogenic immunodetection kit (Invitrogen; catalog# WB7103). The bolts were blocked using Hammersten casein solution for 1 hour and incubated with anti-β-AR antibody (Calbiochem; catalog #PC162) at 1000 fold dilutions for 3 hours. Primary antibody detection was accomplished by incubation in alkaline phosphatase conjugated anti-rabbit IgG at 5000 fold dilutions for 1.5 hours followed by visualization by BCIP/NBT substrate. For proteomic analysis, gel electrophoresis was performed followed by zinc staining as described above. Bands corresponding to 49kDa molecular weight marker were excised, de-stained and submitted to Clemson University Genomics Institute (CUGI) for analysis. The gel bands were digested with trypsin and peptides resulting from this treatment were analyzed by mass spectrometry.
Results

β-adrenergic receptor detection in cells

Out of the total hemocyte population only 4% of the cells labeled positive for the β-adrenergic receptor (β-AR) (Fig.1C). After being stimulated with NA the subpopulation of hemocytes expressing the β-adrenergic receptor formed aggregates and linear chains with intercellular synapses exhibiting extensive cell-cell communication (Fig. 1A & B). Control hemocytes (not stimulated with NA) did not exhibit fluorescence hence NA stimulation appears to be necessary for up regulation of the β-AR in the hemocytes (Fig.1D). Hemocytes stimulated with NA and labeled only with the secondary fluorescent antibody did not exhibit any fluorescence, indicating the specificity of the secondary antibody to the anti-β-AR antibody (data not shown).
Figure 1: NA stimulated β-AR positive cells are a fraction of the total hemocyte population. A: Hemocytes of Crassostrea virginica treated with 10 μM solution of NA and labeled with an anti-β-AR antibody. Cellular aggregates and extensive cell-cell interaction are clearly seen. B: NA treated hemocytes labeled for β-AR, single cell is seen interacting with two other cells, and arrows highlight the point of synapses between the interacting cells. C: Cells labeled for β-AR, approximately 4% of total hemocyte population is positive for the β-AR. Arrows indicate β-AR positive cells. D: Control hemocytes incubated with the anti β-AR antibody yield a negative result. Scale bars: A = 12 μm; B = 4 μm; C = 10 μm; D = 12 μm.

Apoptosis studies

The Cytochrome-c assay detects the release of the mitochondrial inner membrane protein cytochrome-c into the cytoplasm of the cell. The release of which mediates downstream events leading to an apoptotic cascade and programmed cell death. Cytochrome-c release was detected in oyster hemocytes after stimulation with NA (Fig. 2B). We observed about ~4% of the NA stimulated cells are positive for β-AR (Fig.2A) yet ~50% of all the cellular aggregates appeared positive for cytochrome-c (Fig. 2B and 2D).

To further identify the various key points through which the apoptosis cascade is driven by NA stimulation, translocation of phosphatidylserine (PS) from inner leaflet of the plasma membrane to the outer leaflet was detected using the annexin-V assay,
yielding valuable information about the intermediate steps in the cascade. NA stimulation causes ~50% of cells to undergo the PS flip (Fig. 3B) consistent with what was observed with the cytochrome-c assay. NA treated cells form intricate communication networks through cell-cell synapses (Fig. 3D inset box). Cellular aggregation patterns (Fig. 3D) are also similar to those observed in Figure 1A and 2D. To assess the role of NA stimulation on the terminal stages of apoptosis, an Apo-BrdU assay which labels 200 bp long DNA fragments was performed. These DNA fragments are the products of nuclease activity and are detectable when found in the cytoplasm (Fig. 4B). A small percentage of scattered β-AR positive cells are visible in Figure 4A. The cell synapses are clearly visible under bright field illumination (Fig. 4C). The β-AR cells can be seen distributed among the more numerous Apo-BrdU positive cells as shown in the merged image (Fig. 4D).
Figure 2: Cytochrome-c assay detects the onset of apoptosis. This is a three channel image of the NA stimulated hemocytes, β-AR complex (red channel), cytochrome-c in the cytosol (green channel), brightfield and a merged image. A: Of the stimulated population of hemocytes only ~4% of the cells are positive for the β-adrenergic receptor. B: Cytochrome-c release as detected by anti- cytochrome-c antibody, ~50% of the cell population is positive. Cells aggregating in the upper part of the field of view. C: Brightfield image. D: Three channel merge, the distribution of the β-adrenergic receptor positive hemocytes (red & arrows) and hemocytes undergoing apoptosis (green) is evident. Scale bar: D = 10 μm
Figure 3: Annexin-V assay detects the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. This is three channel image of the NA stimulated hemocytes labeled for the β-AR (red channel), PS (green channel), brightfield and merged images. A: NA Stimulated hemocytes seen expressing the β-AR (red channel). B: Apoptotic hemocytes labeled for PS by binding of the fluorescent human anticoagulant annexin-V (green channel). C: Brightfield image, note the cells in the field of view are very thin and barely visible under transmitted light. D: Three channel merge, the co-localization of the red β-AR and green PS to the plasma membrane in some cells is an additive effect resulting in a yellow colored cellular membrane. Arrows showing the cell-cell synapses. Inset the top right of the panel shows close up of the synapses. The stimulation with NA promotes extensive interaction among the β-AR positive hemocytes, and also among the receptor positive hemocytes and other cells of the total hemocyte population. Scale bar: D=5 μm.
**Figure 4:** Evidence of late stages apoptosis by Apo-BrdU assay. A three channel image of the NA stimulated hemocytes labeled for the β-AR (red channel), 200 bp DNA fragments (green channel), and brightfield and merged image. **A:** Arrows indicate the subpopulation of cells positive for the β-AR. **B:** Denatured DNA fragments released from the nucleus into the cytosol labeled with BrdU are visible **C:** Bright field image. Intercellular synapses are visible among cells. **D:** Three channel merge. The distribution of the receptor positive cells and the apoptotic cells is evident. Arrows indicate points of contact between the receptor positive cells and the apoptotic cells. Scale bar D= 10 μm.

Electric cell substrate impedance sensing (ECIS)

As the impedance measurements are taken from the cell membranes, minute changes like receptor up regulation, cell-cell synapses, adhesion and other changes are detectable by ECIS recordings. When the cells settled on ECIS electrodes were stimulated by NA a sudden increase from the basal impedance readings was detected. Impedance readings for subsequent stimuli were always higher than the initial one (Figure 5A). A variety of control experiments were carried out where molluscan PBS and molluscan Ringers solution of different osmolality and ionic concentrations were added to hemocytes adhered to the surface of the array. Control results ruled out the possibility of the response being generated by osmotic or ionic shocks.

To further authenticate the impedance changes generated by NA, a classical β-AR blocking molecule sotalol hydrochloride (SH) was added to the hemocytes. As the
receptors were blocked NA failed to generate a response in the cells and impedance readings remained unchanged. As the binding of SH to the β-adrenergic receptor is reversible in a time dependent fashion, subsequent NA stimuli were able to generate a response in the cells. The magnitude of the response to NA by the SH treated cells also increased over time (Figure 5B)
Figure 5: Electric cell-substrate impedance sensing (ECIS) demonstrates the effects of the NA stimulation on cell membrane dynamics. A: Impedance measurements from well A2 (red peaks), cells stimulated with NA (arrows) at two different time points. Sudden increase in the impedance is observed as a result of NA stimulation. Impedance readings for the second peak are higher than the first one thus indicating the response to the NA increases after the first stimulation. Molluscan PBS was added to control wells A6 (purple line). It failed to generate a response similar to the cells stimulated with NA. B: β-AR were blocked using the beta-blocker, sotalol hydrochloride (SH), thus SH treated cells stimulated with NA do not elicit a response. As the binding of SH to the β-AR is a reversible, subsequent NA stimulation eventually elicits a response.

Immunoblotting and proteomic analysis

SDS-PAGE analysis of proteins from control hemocytes showed that the membrane proteins lack the β-ARs (Fig. 6A), while cells incubated with molluscan PBS containing NA show up-regulation of β-AR in the cell membranes (Fig. 6B). Anti-β-AR antibody recognizes the β-AR complex at the 49 kDa molecular weight marker as seen by western analysis. Proteomics analysis confirmed the 49kDa band matches with β-AR sequence (Fig.7).
Figure 6: The β-AR complex isolated from hemocytes by SDS PAGE followed by Western analysis. A: Coomassie blue stained gel of negative hemocyte controls. The β-AR complex (49 kDa MW) is absent, the three lanes are replicates. B: Coomassie blue stained gel of NA treated hemocytes, β-adrenergic receptor complex is seen at 49 kDa MW, the three lanes represent replicates. C: Western blot, anti-β-AR antibody recognizes the β-AR complex at 49 kDa MW.
**Figure 7:** Mass spectral analysis reveals that receptor complex isolated from hemocytes is homologous to the human β-AR. The low match score is attributed to substitution of moieties in the receptor complex, as the sequence from the oyster protein was queried against a mammalian database.

**Discussion**

A specialized class of oyster hemocytes (β-AR cells) which respond to NA is reported. Although there have been previous studies describing effects of NA on oyster hemocytes (Lacoste, Malham et al. 2001; Lacoste, Malham et al. 2001), a specific subpopulation of cells bearing β-AR receptors were not described.

Observation of a specialized β-AR bearing cell type in oysters is also consistent with predictions made by Coon and Bonar (Coon and Bonar 1987), these authors pharmacologically demonstrated that treatment of oyster larvae with catecholamines such as NA and epinephrine (EPI), caused induction of metamorphosis into unattached juvenile oysters. Although the mechanism through which NA produces unattached juvenile oysters was not well understood, it was predicted that there could be a specialized class of cells bearing adrenergic receptors, mediating effects of catecholamines through a paracrine pathway. The identity of the β-AR has been described in this study by immunofluorescence, immunoblotting and mass spectrometric analysis. The β-AR cells comprise only ~4% of the total circulating hemocytes population yet after NA stimulation ~50% of the hemocytes undergo apoptosis. As the β-
AR cells are always seen in proximity of hemocytes undergoing apoptosis and as the general population of hemocytes do not bear receptors for NA, it is likely that the β-AR cells mediate effects of NA through a paracrine pathway. Furthermore presence of extensive cell-cell synapses between β-AR cells and apoptotic hemocytes was observed (Fig. 1A-B, 3D inset box & 4D).

β-AR cells might be similar to chromaffin cells which are catecholamine bearing cells found in the oyster heart and release NA when the oysters are subjected to mechanical stress (Lacoste, Malham et al. 2001). Molecular identity of the signaling molecule in this NA stimulated-β-AR cells mediated apoptotic pathway remains unknown. The β-AR cells could be mediating apoptosis by release of variety of signaling molecules. Molluscan apoptotic mechanisms appear to be highly conserved and analogous to better known vertebrate systems (Ottaviani and Franceschi. 1996). Catecholamines induce apoptosis in human melanocytes by generating reactive oxygen species (ROS) (Chu, Liu et al. 2006). NA has also been shown to induce apoptosis in rat cardiomyocytes via release of tumor necrosis factor-α (TNF-α) and caspases-2,-3,-6, and -9. The activity and secretion of TNF-α and caspases was completely negated when NA-stimulated cardiomyocytes were treated with antioxidants, indicating a crucial role played by ROS in the NA mediated apoptosis (Fu, Ching-Shiang et al. 2004). Thus ROS could be one of the signaling molecules released by the β-AR cells upon NA stimulation. Additional research is needed to identity of these death effectors released by the β-AR cells in NA stimulated apoptosis cascade. Juvenile oysters subjected to mechanical stress or injected with NA become highly susceptible to Vibrio splendidus infections (Lacoste,
This could perhaps be explained by the paracrine pathway of apoptosis mediated by the β-AR cells, resulting in a down regulated immune state, leaving the oyster susceptible to bacterial infections.

Our results show that NA stimulated apoptosis of in oyster hemocytes is detectable from earliest onset to the last stages. ECIS studies have revealed that the NA receptor is stimulated within a few seconds of agonist addition, downstream cellular effects occur within minutes and the adrenergic receptor complex is reset within 30 minutes. ECIS studies rely on minute impedance changes taking place on the cell membrane of hemocytes. Upon NA stimulation a major increase in impedance was detected indicating changes in cell membrane as well as the cytoskeleton. These impedance changes brought on by NA stimulation are blocked by classical β-AR blocker sotalol hydrochloride (SH), proving that membrane and cytoskeletal changes in hemocytes are in response to NA stimulation. This is a first report of impedance studies being performed on oyster hemocytes.

Exploration of surfaces by invertebrate larvae is an important step leading to attachment and metamorphosis into juveniles (Crisp 1974; Pawlik 1992; Hadfield 1998). Before settling, oyster veliger larvae and barnacle cypris larvae investigate surfaces using exploratory structures bearing an array of sensory cells (Clare 1995; Zimmer-Faust, Tamburri et al. 1996; Walters, Miron et al. 1999). Stimulation with NA and other catecholamines has been shown to cause loss of exploratory behavior in oyster larvae, leading to metamorphosis into juvenile oysters without attachment to a substratum, a phenomenon used by the oyster hatcheries for commercial production of cultchless
oysters (Coon, Bonar et al. 1985; Coon, Bonar et al. 1986; Coon and Bonar 1987; Coon, Fitt et al. 1990). Barnacle cypris larvae stimulated with NA also respond in a similar manner and lose their exploratory behavior, metamorphosing into unattached juvenile barnacles (see chapter 2). Signaling molecules attached to polymer surfaces have been shown to exert site specific responses on adhering cells (Hersel, Dahmen et al. 2003; Harris, Kutty et al. 2006). The act of exploration of surfaces before settling is a crucial process in many invertebrate larvae, a process which can be taken advantage of in engineering fouling resistant coatings. It is hypothesized that surfaces with covalently tethered NA or other catecholamine analogues could induce a stress response in the invertebrate larvae. The perception of presence of an ‘unfavorable’ signaling molecule by an array of sensory cells present on the exploratory structures of the larvae would deter the larvae from settling on such a surface. Catecholamines induced neuroendocrine changes and seem to divert the organism’s energy resources away from physiological functions such as growth, reproduction and immunity. The metabolic and behavioral adaptations help the animal to overcome the threat to survival (Ottaviani and Franceschi. 1996; Maule and Vanderkooi. 1999; Lacoste, Jalabert et al. 2001). This ability of catecholamines to induce a myriad of physiological changes including disruption of settling behavior in molluscan larvae without exerting any harmful effects, gives precedence for studying catecholamines as fouling deterring molecules. Assays and techniques developed in this study will be applied to further investigate the intriguing effects of catecholamines at cellular level in the marine invertebrate larvae. This study focuses on understanding marine invertebrate fouling from a cellular perspective,
understanding of which will allow engineering of fouling deterrent coatings which would
be as effective as they are environmentally benign.
CHAPTER THREE

NORADRENALINE INHIBITS SETTLING BEHAVIOR AND CEMENTATION IN THE CYPRIS LARVAE OF THE STRIPED BARNACLE, *Balanus amphitrite*

Abstract

The effect of the neurotransmitter noradrenaline (NA) was investigated on the cyprids of the barnacle *Balanus amphitrite*. One day old cyprids were challenged with micro-molar concentrations of NA ranging from 30-100 μM and assayed over 4 days. Multi-dimensional imaging was carried out with Zeiss discovery V12 and Nikon AZ100 macro-stereoscopes and samples were preserved for SEM analysis at 24 hour intervals. ‘Searching behavior’ of the cyprids was lost after NA challenge and the larvae failed to cement to the substratum. A considerable delay in cyprid-adult ecdysis was noted. Remnants of cyprid organs such as the antennules were clearly visible in metamorphosed juveniles. Juvenile barnacles appeared to be normal as compared to the controls.
Introduction

Benthic marine invertebrates have planktonic larvae that remain in the water column for variable periods of time before settling on suitable substrata. Understanding the internal and external factors which mediate marine invertebrate larval attachment and metamorphosis is important for developmental biology, population and community ecology as well as biofouling and aquaculture (Dahms, Jin et al. 2004). Barnacles, like most other invertebrates, advance through a complex program of larval development leading to metamorphosis into the sessile juvenile stage. Larval development involves six planktonic nauplius stages followed by the lecithotrophic cypris stage. The cyprid is specialized to locate a suitable site for attachment and metamorphosis (Darwin 1854). Barnacle cypris larvae investigate surface using an array of sensory structures present on the third and the fourth segment of the cyprid antennules (Visscher 1928; Nott and Foster 1969; Clare, Freet et al. 1994; Clare and Nott 1994). Chemical stimuli associated with a suitable habitat induce stereotypes of reversible settlement behaviors on or near the substrate which culminate in irreversible metamorphosis (Coon, Fitt et al. 1990).

The striped barnacle *B. amphitrite* is a cosmopolitan species of great biofouling importance (Bishop 1950) and is distributed worldwide in warm and temperate seas. *B. amphitrite* is found from Massachusetts in North America to Mediterranean, West Indies, South Africa to the Philippines archipelago (Zullo 1963). Thus *B. amphitrite* has become the centre of focus for experimental biofouling research (Clare and Høeg 2008).
Biogenic amines are neurotransmitters which influence a variety of behavioral and physiological processes in both vertebrates and invertebrates (Isoai, Kawahara et al. 1996). Biogenic amines have been cyprid settlement in *B. amphitrite*. L-DOPA and dopamine have been shown to induce metamorphosis prior to attachment in *B. amphitrite* (Kon-Ya K, Shimidzu N et al. 1994; Kon-Ya K and Endo 1995). Lisuride a serotonergic agonist/antagonist and dopaminergic agonist has been shown to inhibit cyprid larval settlement and promote larval searching behavior (Yamamoto, Akiko Tachibana et al. 1996). The α-adrenoreceptor agonist medetomidine and clonidine have been shown to inhibit *Balanus improvisus* cypris larval settlement. (Dahlström, Mårtensson et al. 2000).

A variety of imidazoline/guanidinium compounds which have overlapping α-adrenoreceptor and imidazoline binding sites in vertebrates have been shown to inhibit cypris larval settlement in *B. improvisus* (Mia Dahlström, Lindgren et al. 2005). The α-adrenergic antagonist idazoxan and phentolamine have been shown to inhibit settlement of *B.amphitrite* cypris larvae causing mortality (Dahms, Jin et al. 2004). A G-protein coupled receptor (GPCR) has been cloned from the genomic library of *B.amphitrite*, the receptor protein has been shown to have 36.9% homology to the human α2-adrenergic receptor (Isoai, Kawahara et al. 1996). These are some of the studies which overwhelmingly indicate involvement of andrenoreceptor signaling in settlement and metamorphosis in barnacles.

Catecholamines have also been shown to inhibit settlement in bivalve mollusks of biofouling and aquaculture importance. Noradrenaline (NA) inhibits larval settlement of Pacific Oyster, *Crassostrea gigas* and the Eastern Oyster, *Crassostrea virginica*. NA
treated larvae of *C. gigas* and *C. virginica* forgo the settlement behavior and metamorphose without cementing to a substratum (Coon, Bonar et al. 1985; Coon, Bonar et al. 1986; Coon, Fitt et al. 1990).

The adrenoreceptor antagonists idazoxan and phentolamine inhibit larval settlement and cause mortality in the larvae of Bryozoan, *Bugula neritina* (Phylum: Ectoprocta, Class: Gymnolaemata) and the tube worm, *Hydroides elegans* (Phylum: Annelida, Class: Polychaeta) both species are of biofouling importance.

Owing to their broad ranging effects on inhibiting the larval settlement in many phyla of benthic marine invertebrates of biofouling importance, adrenoreceptor agonist/antagonists are promising as novel antifouling compounds. Although there have been extensive efforts at screening various compounds, these studies have majorly focused on cyprid settlement assays to understand effects of andrenoreceptor compounds on inhibition of cyprid settlement (Yamamoto, Akiko Tachibana et al. 1996; Yamamoto, Akiko Tachibana et al. 1998; Yamamoto, Katsuhiko Shimizu et al. 1999; Dahlström, Mårtensson et al. 2000; Dahms, Jin et al. 2004; Mia Dahlström, Lindgren et al. 2005). Little is known about mechanisms behind larval settlement and effect of such compounds on cyprid-barnacle metamorphosis.

To develop a better understanding of adrenoreceptor agonists on cerripedian metamorphosis, this study evaluated the effects of noradrenaline on *B.amphitrite* cypris larvae using advanced light microscopy and scanning electron microscopy. Cyprid metamorphosis was followed 96 hours (hr) post NA treatment and observations were made every 24 hr. The goal of the study was to observe structural differences between
NA induced cyprid-barnacle metamorphosis and metamorphosis under normal conditions.

Materials and methods
Cypris larval culture

One day old cyprid larvae of the barnacle *Balanus amphitrite* were obtained from the Rittschof Laboratory at Duke University Marine Laboratory (Beaufort, NC). The osmolality of sea water in which the larvae were reared was checked using a micro-Osmometer (Advanced instruments) and salinity was checked using a salinity refractometer (Aquatic eco-systems Inc). The osmolality was measured in the range of 900-960 mOsm and salinity was measured at 32-34 ppt. The salinity and osmolality values of the artificial sea water (ASW) used for the larval experiments were matched to the natural sea water in which the larvae were reared.

Noradrenaline (NA) treatment

One experimental group of larvae was incubated in 35mm petri dishes (Thermo-Fisher Inc.) with ASW containing 30, 50, 70, 100 μM of NA. This particular concentration range for NA was chosen as it is known to induce metamorphosis without attachment in larvae of the Eastern Oyster (*Crassostrea virginica*) and the Pacific Oyster (*Crassostrea gigas*) (Coon, Bonar et al. 1985; Coon, Bonar et al. 1986). Twenty larvae
were dispensed in each petri dish and three replicate were used per concentration of NA tested.

Larvae were incubated with ASW containing NA for 24 hours, after which the larvae were washed twice with ASW free of NA and incubated in new petri dishes containing fresh ASW without NA.

The second experimental group was flash treated with NA. As described earlier, 20 cyprids were dispensed in each petri dish containing ASW with 30, 50, 70, 100 μM of NA. Three replicates were used per concentration of NA tested. The cyprids were incubated in ASW with NA for three hours, then washed with fresh ASW free of NA and incubated new petri dishes with ASW free of NA. Each experimental group was also compared to negative (NA free) controls. Ninety-six hours post NA treatment attached and unattached cyprids were counted for each petri dish. Percent attached and unattached cyprids were determined by averaging the counts from the three replicates used per concentration. Cyprid-barnacle metamorphosis was followed for both the experimental groups as well as controls for 96 hours.

**Light microscopy**

Cyprid ecdysis and metamorphosis to juvenile barnacle was followed for 96 hours. Observations were made at 24 hr, 48 hr, 72 hr and 96 hr using the Zeiss Discovery V-12 stereo microscope equipped with a Zeiss Axiocam-MRC5 camera (Carl Zeiss Inc.) and a Nikon AZ-100 stereo-macroscope equipped with a Nikon DXM-1200C camera.
For all the treatments and controls, representative images were obtained from every replicate petri dish. At each of 24 hr observation points along the experiment, representative specimens were preserved for scanning electron microscopy (SEM) analysis as described below.

**Scanning electron microscopy (SEM)**

At each of the 24 hr time points, representative specimens were subjected to primary fixation using mixture of 2.5% glutaraldehyde and 2% paraformaldehyde (Electron Microscopy Sciences EMS, Hatfield, PA) made up in 100mM sodium cacodylate buffer (EMS) for 2 hours. Samples were then washed three times for 15 minutes each. Secondary fixation followed with a 1% solution of osmium tetroxide (EMS) made up in 100 mM sodium cacodylate buffer for 45 minutes. Following secondary fixation, samples were washed with deionized water three times for 15 minutes each. The samples were dehydrated through a graded ethanol series of 25%, 50%, 75%, 80%, 90%, and 100%. In the graded series, dehydrations between 50%-100% ethanol were carried out at 0°C to minimize extraction. Samples were transferred to a chamber of critical point dryer (CPD) in ethanol. Samples were then subjected to three washes of liquid CO$_2$, five minutes each to remove ethanol from the sample. Critical point drying was carried out at 1072psi at 31°C. After CPD samples were mounted on aluminum stubs using double sided carbon tape, and sputter coated using a Denton sputter coater with a
gold-palladium target. Samples were imaged using Hitachi Field Emission-S4800 scanning electron microscope (Hitachi high technology Inc).

Results
Analysis of cyprid metamorphosis after NA treatment using light microscopy

Following NA treatment (either by flash induction or by incubating the cyprids in ASW containing NA) cyprids stopped swimming and sank to the bottom of the petri dishes with momentary movements of the thoracopods and the antennules. The noted “searching behavior” (Crisp and Meadows 1962; Crisp and Meadows 1963; Crisp 1974) of the cyprids was also lost after NA treatment and the cyprids did not attach or cement to substratum. Approximately 70% of the NA treated cyprids did not attach to the substratum and metamorphosed into floating juvenile barnacles while ~30% of NA treated cyprids settled and metamorphosed into juvenile barnacles (Fig. 4). Approximately 80% of control cyprids settled within 24 hours and metamorphosed into juvenile barnacles.

Twenty–four hours (hr) post NA treatment, the cyprids did not display any signs of ecdysis. The onset of ecdysis was observed between 24 and 48 hr post NA treatment. Metamorphosing juvenile barnacles were seen encased in bivalve cyprid carapace (Fig 1A-D). The pumping action of the thorax similar to beating of cirri was also observed (Glenner and Høeg 1993). Seventy-two hours post NA treatment the metamorphosing barnacles displayed recognizable features such demarcations between the base plates and
the opercular plates (Fig. 1C-D). Some ecdysing barnacles were still encased in cyprid carapaces while others had shed the bivalve carapace. The cyprids that had shed carapaces were presumed to have ‘grown’ out of it as opposed to have shed carapace voluntarily as observed in untreated attached cyprids (Glenner and Høeg 1993). A well demarcated operculum from the base plate was observed in juvenile barnacles (Fig. 1C).

Ninety-six hours post NA treatment, the operculum with tergal and scutal plates was well demarcated from the wall plates (Fig. 1E). In contrast, control barnacles were seen fully calcified with well recognizable wall and opercular shell plates at around 24-48 hr post settlement (Fig.1F). Although a great deal can be learnt about cyprid metamorphosis using light microscopy, need for an ultrastructural technique such scanning electron microscopy (SEM) is evident for better understanding effect of NA on cyprid ecdysis.
**Figure 1:** Effect of NA on the attachment and metamorphosis of cypris larvae. 

A-B) Cyprid larva ecdysing into unattached juvenile barnacle, 48 hours post NA treatment. Ecdysing juvenile barnacle (green outline) remains encased inside the carapace of the metamorphosed cyprid (yellow outline). White arrow: Base plate of the juvenile barnacle. Black arrow: Exoskeleton of the thoracopods of the cyprids. 

C-D) Unattached floating juvenile barnacle (green outline) still encased in cyprid carapace (white arrow) with discernable base plate (black arrowhead) and operculum (black arrow), 72 hr after NA treatment. 

E) Unattached juvenile barnacle 96 hr post NA treatment displays a well demarcated operculum (black arrow) from the wall plates (white arrow). 

F) Control, calcifying attached barnacle. Ro: rostrum. Ca: carina plate, Wp: wall plate. After (Glenner and Høeg 1993). Scale bar: A=100 μm, C= 100 μm, E= 100 μm, F=100 μm
Analysis of cyprid metamorphosis following NA treatment using SEM

SEM analysis of cyprid ecdysis post NA treatment revealed key details. Twenty-four hours post NA treatment intermediate stages between the cyprid and juvenile barnacles were observed (Fig. 2A-E). The metamorphosing barnacles were encased in the bivalve cyprid carapace (Fig. 2A-B). Twenty-four hours post NA treatment, although some of the features of the adult stage such the cirri and the base plate were evident, intact antennules of the cyprid with well recognizable features were seen retained in the metamorphosing barnacle (Fig. 2C-D). The ecdysing barnacles were covered with a soft cuticle without any evidence of calcification (Fig. 2E). The soft cuticular base plate of the metamorphosing barnacle displayed both cyprid antennules with four antennular segments. Metamorphosing barnacles also possessed the cuticular basal hair which is the hallmark of the juvenile barnacle stage (Fig. 2E). Forty-eight hours post NA treatment, metamorphosing barnacles, some still encased in the bivalve cyprid carapace, displayed a differentiating operculum and a base plate (Fig. 3A). Seventy-two hours post NA treatment, the operculum could be discerned in to the terga and the scuta (Fig. 3B).

Majority of the juvenile barnacles observed at the 72 hr had lost the cyprids carapace. The barnacles were still covered with a soft cuticle, at this point the metamorphosing barnacles did not display any remnants of the cyprid stage.

At 96 hr the barnacles appeared calcified with an operculum well demarcated from the wall plates (Fig. 3C-D). The incipient wall plates, although discernable in to carina and rostra (Fig. 3C-D) were not completely developed. At 96 hr, centre of the
juvenile barnacle base plates contained remnants of the cyprid antennules (Fig. 3E), the third segment of the antennules was identifiable. The base plates had calcified yet there were no signs of adult barnacle glue secretion.
**Figure 2:** SEM analysis of ecdysing cyprid larvae 24 hours post NA treatment. **A)** Intermediate step between a cyprid larvae and a juvenile barnacle, the ecdysing juvenile (black arrow) is still encased in the cyprid carapace (white arrow). **B)** Metamorphosing juvenile barnacle still attached to the cyprid carapace (white arrow). Cyprid antennules on base plate of the juvenile barnacle are clearly visible. **C)** Metamorphosed juvenile barnacle with recognizable features from the adult stage such as the cirri (black arrow), and a developing base plate (Bp). The unattached juvenile still retains antennules from the cyprid stage with recognizable features (white arrow). **D)** Magnified view of the cyprid antennules from inset box in fig. 2C. The cyprid antennules retained in the metamorphosing juvenile barnacle bears all the recognizable features from the cyprid stage. S2: second antennular segment. S3: third antennular segment, the attachment disc. VM: velum bordering the attachment disc. SS: Sub terminal setae of the fourth antennular segment. TS: terminal setae of the fourth antennular segment. P2: postaxial seta 2 of the second antennular segment. **E)** Close up view of the base plate of an unattached metamorphosing juvenile barnacle. 24 hours post NA treatment, the base plate remains to be calcified. Progression of ecdysis from cyprid to juvenile is evinced by the presence of cuticular basal hair (BH). The cyprid antennules are retained in on the base plate with clearly discernable features. S1: First antennular segment. S2: Second antennular segment. S3: Third antennular segment. S4: Fourth antennular segment. P2: Postaxial seta 2. P3: Post axial seta 3. TS: Terminal seta. BH: Cuticular basal. After (Nott and Foster 1969; Glenner and Høeg 1995). Scale bar; C=100 μm, D= 10 μm.
**Figure 3:** SEM analysis of unattached metamorphosing cyprids 48, 72, 96 hours post NA treatment. A) 48 hour post NA treatment. Ecdysing barnacle with recognizable features such as the operculum (white arrow) and developing base plate (black arrow). The developing barnacle can be seen encased in the bivalve cyprid carapace. B) 72 hours post NA treatment. The pinhead barnacle form is evident with discernable features such as opercular plates, scuta (SC) and terga (TE), although still uncalcified and encased in a soft cuticle, possessing cuticular basal hair (black arrow). C) 96 hours post NA treatment. A metamorphosed barnacle is seen without any traces of the cyprid stage. Calcified shell plates such as the carina (Ca), rostrum (Ro) and wall plates (Wp) are well demarcated from the opercular shell plates such as the terga (TE) and scuta (SC). The cirri (white arrow) can be seen protruding from the operculum. D) 96 hours post NA treatment. The barnacle is fully calcified with well demarcated shell plates and a closed operculum. SC: scuta. TE: terga. Wp: wall plates. E) 96 hours post NA treatment. Calcified base plate of an unattached barnacle; remnants of the cyprid antennules can be seen at the centre of the base plate (black arrow). Short black arrows indicate growth increments around the periphery of the base plate.
Unsettled and unattached cyprids

Settled cyprids

Effect of Noradrenaline on the settling and cementation of *B. amphitrite* cypris larvae
Figure 4: Effect of noradrenaline on the settling behavior of *B. amphitrite* cypris larvae. Approximately 70% of NA treated cypris larvae did not display settling behavior and metamorphosed into juvenile barnacles without attaching to the substratum. Approximately 30-35 % of NA treated cypris larvae settled and metamorphosed into juvenile barnacles. Concentrations ranging from 30 µM-100 µM did not have display variations on inhibiting the cyprids settling behavior.
Discussion

NA treated cypris larvae of the barnacle *B. amphitrite* failed to attach to the substratum and metamorphosed into juvenile barnacles. Metamorphosis without attachment was observed in both experimental groups, independent of time of incubation. Thus NA is able to stimulate metamorphosis in cyprids even after 3 hr of incubation. In a similar study (Coon, Bonar et al. 1986), larvae of the Eastern Oyster *C. virginica* and the Pacific Oyster *C. gigas* were induced to metamorphose without attachment by NA and epinephrine (EPI) treatment in as little as 1 hr. The authors of that study did not see a statistical difference in NA induced larval metamorphosis in larvae incubated in NA for 1 hr versus larvae incubated in NA for 24 hr. Approximately 70% of cyprids treated with 30 µM-100 µM of NA did not display searching behavior and metamorphosed into unattached juvenile barnacles while approximately ~30% of NA treated cyprids attached and metamorphosed into juvenile barnacles (Fig. 4). Concentrations from 30 µM-100 µM showed little variations in the settlement inhibiting effect mediated by NA on the cypris larvae.

Furthermore α2-adrenoreceptor agonists medetomidine and clonidine have also been demonstrated to inhibit settlement in the cypris larvae of *B. improvisus* (Dahlström, Mårtensson et al. 2000; Mia Dahlström, Lindgren et al. 2005).

Exploration of surfaces by invertebrate larvae is an important step leading to attachment and metamorphosis to juveniles (Crisp 1974; Pawlik 1992; Hadfield 1998). Before settling, oyster veliger larvae and barnacle cypris larvae both investigate surfaces
using exploratory structures bearing an array of sensory cells (John A. Freeman 1979; Clare 1995; Zimmer-Faust, Tamburri et al. 1996; Walters, Miron et al. 1999). The ‘noted searching’ behavior of the cyprids was lost post NA treatment and the larvae sank to the bottom of the petri dishes and began the process of metamorphosis. Veligers of the *C. virginica* and *C. gigas* have also been shown to lose the searching behavior post NA treatment (Coon, Bonar et al. 1985). Under normal circumstances the initiation or delay of the premolt is under the cyprid control and can be delayed in some regions of the cyprid epidermis (John A. Freeman 1979). It is speculated that since the muscles which regulated the thoracic appendages in cyprids are anchored to the dorsal epidermal cells (Walley and Rees 1969), this region only undergoes apolysis only after the settlement process. Apolysis of this region prior to settlement would render the cyprids unable to swim (John A. Freeman 1979). It can be hypothesized that NA stimulation supersedes the cyprid control of the molting cycle resulting in apolysis in all the epidermal regions. Thus apolysis of the dorsal epidermis cells where the muscles controlling the thoracic appendages are anchored could lead to the loss of the searching behavior as the cyprids are unable to swim.

The cement gland in the cyprids of the barnacle *Megabalanus rosa* has been shown to be under the control of catecholaminergic neurons. Cement exocytosis was observed by incubating the explanted cement glands in catecholamine solution including NA (Okano, Shimizu et al. 1996).

There was no evidence of cement exocytosis following NA treatment this was also shown by absence of cement secretion through the 3rd antennular segment (Fig 1A-E
Fig. 2A-E). Thus although the explanted cement glands might respond to NA in-vitro, this is not the case when the cyprid nervous system is intact. It appears that NA acts though the nervous system in a way that countermands cement secretion. Thus explaining the apparent absence of cement in the NA treated larvae.

NA treatment also appears to delay the cyprid ecdysis and the following metamorphosis to juvenile barnacles. Glenner et al (1993) followed the metamorphosis of four species of barnacles using scanning electron microscopy. Ecdysis is defined as the shedding of cyprids carapace thus marking the transition from the cyprid to the first instar of the juvenile barnacle (Glenner and Høeg 1993). Under natural conditions ecdysis has been observed 4-6 hr after initial attachment in B. amphitrite cyprids (Glenner and Høeg 1993). In this study NA treatment caused the loss of cyprid searching behavior and prevented the cyprids from settling or attaching to the substratum. Ecdysis was observed 24 hr post NA treatment. Since the cyprids were not attached to the substratum (even after ecdysis) the juvenile barnacles retained the cyprid carapaces as long as 48 hr post treatment (Fig. 2A & Fig. 1A-D). Under natural circumstances the muscular contractions of the soft body moves the juvenile instar slowly around the pivot point of the cyprid cement aiding the release of the cyprid carapace from the ecdysing barnacle (Glenner and Høeg 1993). Since NA treated cyprids are unattached, the cyprid carapaces are retained and not discarded in the manner described above. The juvenile barnacles eventually grew out of the cyprids carapaces around 48 hr, post NA treatment. Under natural conditions, 4-6 hr post ecdysis no traces of the cyprid stage are observed (Glenner and Høeg 1993). As the control barnacles settled and cemented to the substratum, no remnants of the
cyprid stage were observed at 24 hr observation point. While the remnants of the cyprid stage were observed up to 24-48 hr in NA treated cyprids (Fig. 2A-E). The cyprid antennules comprised of four segments were observed in great detail (Fig. 2E). Putative histolysis of these cyprid antennules occurred between 48-72 hr post NA treatment. At the 72 hr observation point remnants of the cyprid stage had disappeared (Fig. 3B). Although at the 96 hr observation, the centre of the base plates of the juvenile barnacles displayed vestiges of the cyprid antennules (Fig.3E). Glenner et al (1993) observed the incipient the shell plates 5-6 hours after ecdysis in B. amphitrite. The incipient shell plates were observed between 48-72 hours post NA treatment.

The pathway through which NA is able to induce metamorphosis independent of settlement behavior still remains to be investigated. A gene encoding a putative G-protein coupled receptor (GPCR) has been cloned from the genomic library of B. amphitrite (Isoai, Kawahara et al. 1996). The protein encoded by this gene has a consensus G-protein binding motif (Ala-Ile-Ser-Leu-Asp-Arg-Tyr-Leu-Ala) and is closely related to the human α2-adrenergic receptor with a 36.9% identity in 409 amino acid overlap (Isoai, Kawahara et al. 1996). Thus it can be hypothesized that NA mediates its effects on the cyprids through a GPCR possibly a member of the adrenergic receptor family. Because NA induces metamorphosis without the settlement, its acts on receptors which probably function later or through a different pathway in the cascade of settlement and metamorphosis (Coon, Bonar et al. 1986; Coon and Bonar 1987). It is also possible that NA or NA like compounds occur naturally in the barnacles, post attachment, which
initiate metamorphosis (Coon, Bonar et al. 1985) the source and nature of such molecules remains to be investigated.

It is not clear whether the putative α or β adrenoreceptors control the metamorphosis directly or indirectly at the tissue level in *B. amphitrite*. NA could stimulate the receptors mediating the release of a morphogen from some neurohormonal site, the released morphogen could be actually active at the tissue level (centralized receptor theory) (Coon and Bonar 1987). Such factor could be a hormone e.g. crustecdysone (β-ecdysone, ecdysterone, 20-hydroxyecdysone), a steroid hormone similar to ecdysone which controls molting in arthropods (Tighe-Ford and Vaile 1972). These receptors might be located on a limited number of cells, analogous to control of neurosecretory release of growth hormone in *Lymnaea* (Coon and Bonar 1987). Alternatively, the receptors could be located on the target tissues and receive the adrenergic stimulus (NA) which would be released as a hormone into the circulatory system of the larva (Coon and Bonar 1987). Substantial amounts of endogenous NA and possibly EPI, have been demonstrated in *C. gigas* as evidence that stimulation of α-adrenergic receptors is involved in the natural metamorphic process (Coon and Bonar 1986).

Whether the receptors are located centrally or peripherally, they are not restricted to hormonal control, but could also mediate metamorphic control acting through direct neural stimulation. The nervous system of the cypris larva of the *B. amphitrite* contains fewer than 2000 neurons and is well organized for coordinating response to settlement cues (Harrison and Sandeman 1999). Okano et al (1996) have shown that the cement
glands of cypris larvae of *B.amphitrite* are under the control of catecholaminergic neurons and respond to NA. Catecholamine containing cells have also been documented in the larvae of the two bivalve families, the *Pectinidae; Placopecten magellanicus* and the *Mytilidae; Mytilus edulis* (Croll, Jackson et al. 1997). The catecholamine bearing cells in these larvae have been documented to be in the fibers in the velum, foot, and mantle and around the mouth. Catecholamine bearing cells in the foot are connected to the pedal ganglia and the abdominal ganglia by catecholamine bearing fibers, perhaps catecholaminergic neurons (Croll, Jackson et al. 1997).

Thus it is tempting to speculate that NA acts directly on the nervous system of the cypris larvae of the *B.amphitrite* and is able to mediate metamorphosis without attachment. Such mechanism is also postulated by Coon et al (1987) to explain NA induced metamorphosis in larvae of *C. gigas*.

The mechanism through which NA induces metamorphosis must account for the multiplicity of changes which occur, including histolysis, histogenesis, proliferation, biochemical and physiological changes. A study similar to Croll (1997) could shed insights on locations of the putative catecholamine bearing cells and tissues in the cyprids larvae of *B. amphitrite*. It is now possible to isolate specific cells from histological sections using laser capture microdissection, such cells and tissues could be then subjected to genomic and proteomic analysis (Rachel A. Craven 2001; Silasi, Alvero et al. 2008) to identify specific genes and proteins involved in NA mediated metamorphosis of cyprids. Such a study will elucidate receptors and the signal transduction mechanisms involved ecdysis and metamorphosis in barnacles which are so poorly understood.
The \( \alpha \)-adrenergic antagonists idazoxan and phentolamine have been shown to inhibit settlement cyprids of *B. amphitrite* (Arthropoda), *Bugula neritina* (Ectoprocta) larvae, and larvae of the *Hydroides elegans* (Annelida) in a concentration and taxon-dependent manner (Dahms, Jin et al. 2004).

These results correlated with results obtained in this study suggest involvement of adrenergic receptor signaling in cyprid larval attachment and metamorphosis. Stimulation of these signal transduction pathways by NA leads to metamorphosis without attachment while blocking of these pathways by antagonists manifests detrimental effects on the cypris larvae. Thus adrenergic receptor agonists such as NA could perhaps induce metamorphosis without attachment in the Bryozoan and annelidan larvae, a pharmacological study similar to Coon et al (1987) is warranted to further explore and identify catecholamine binding receptors present in these organism. Yamamoto et al. (1996-1999) examined the effect of blockers against serotonin uptake and the adrenergic antagonist phentolamine on the settlement and metamorphosis of *B. amphitrite* cypris larvae (Yamamoto, Akiko Tachibana et al. 1996; Yamamoto, Akiko Tachibana et al. 1998; Yamamoto, Katsuhiko Shimizu et al. 1999).

Presence of adrenoreceptors in arthropods (Yamamoto, Katsuhiko Shimizu et al. 1999; Dahms, Jin et al. 2004), mollusks (Coon and Bonar 1987), Bryozoans and annelids (Dahms, Jin et al. 2004) may indicates that these receptors appeared early in evolutionary history and may be present in other marine invertebrate phyla. The Role of biogenic amines such as NA is evident in settlement and metamorphosis in many marine invertebrate larvae of biofouling importance. Thus precedence should be given to
understanding the various signal transduction pathways affected by these neurotransmitters. Taking such an approach would facilitate developments of fouling deterrent compounds which inhibit the settlement of invertebrate larvae without exerting any deleterious effects on marine environment. Furthermore uninformed use of such compounds without fully understanding the multitude of effects mediated by them could lead to disastrous effects on the marine ecosystems comparable to the devastating impact of TBT on the world oceans.

Settlement and metamorphosis in many marine invertebrate larvae can be experimentally separated using catecholamines such as NA thus providing a unique insight in process of marine invertebrate larval development. Taking such a biological and organism driven approach to biofouling where the pathways involved in fouling are understood and targeted, will lead to development of safe and efficient fouling deterrence strategies as opposed to simply screening and blindly implementing organic compounds in polymeric coatings.
CHAPTER FOUR
COVALENTLY CONJUGATED NORADRENALINE MOLECULES INDUCE
APOPTOSIS IN THE HEMOCYTES OF EASTERN OYSTER *Crassostrea virginica*

Abstract

Noradrenaline (NA) molecules were covalently conjugated to 2-hydroxyethyl methacrylate (HEMA) and methacrylic acid (MAA) polymer surfaces. Amine groups on the NA molecules were covalently attached to the polymer surfaces using carbonylimidazole chemistry. Annexin-V assay demonstrated that NA conjugated polymer surfaces induced apoptosis in adhering hemocytes of the Eastern Oyster *Crassostrea virginica*. Cytoskeletal structure of the hemocytes underwent deterioration upon adhering to NA conjugated polymer surfaces as demonstrated by phalloidin staining and scanning electron microscopy. Control HEMA and MAA polymer surfaces did not induce apoptosis in adhering hemocytes. Cytoskeleton of hemocytes adhering to control HEMA and MAA surfaces did not show any deterioration. A novel fouling deterrence strategy based on NA conjugated polymer surfaces is discussed.
Introduction

Biofouling is the unwanted accumulation of bacteria, algae, plants and marine animals on submerged structures in marine environments. Biofouling leads to aesthetic and structural disintegration and increased surface roughness (Olsen, Pedersen et al. 2007). For ships, the increase in surface roughness translates into high frictional resistance leading to increase in weight, potential reduction in speed and loss of maneuverability. This leads to increase in fuel consumption which can be up to 40% and the cost of the voyage can increase as much as 77% (Yebra, Kiil et al. 2004) as well as increasing harmful emissions. Biofouling also leads to: i) Deterioration of coatings, leading to corrosion, discoloration and change in the electrical conductivity of the surface. ii) Introduction of non-native species to new geographical regions. iii) Increase in the dry-docking cycles of ships resulting in strategic and monetary loss as well as generation of hazardous waste during the cleaning and re-painting process. (Yebra, Kiil et al. 2004).

Marine biofouling has been combated throughout maritime history. From protecting the hulls with copper and lead sheathing to coating ships with pitch, tar, brimstone and tallow, antifouling coatings have been plentiful and diverse (Yebra, Kiil et al. 2004; Olsen, Pedersen et al. 2007). Since the 1950s triorganotin biocide based paints were used as antifoulants (AF). Tributyltin (TBT) incorporated in either soluble or insoluble matrix paints or self polishing coatings (TBT-SPC) was used extensively as an effective broad spectrum antifouling coating beginning early 1960s. The wide spread use
of these paints is estimated to cover 70% of the present world fleet (Yebra, Kiil et al. 2004). Unfortunately TBT-SPC paints have an adverse effect on the environment. TBT leached out of paints even at extremely low concentrations causes devastating effects on the marine biota. At concentrations of 20 ng/l it causes shell deformities in the Pacific Oyster *Crassostrea gigas* (Yebra, Kiil et al. 2004). At even lower concentrations of 1 ng/l it causes imposex, imposition of male genitalia on females of the dog-whelk, a marine snail (Folsviksrk, Berge et al. 1999; Yebra, Kiil et al. 2004). Consequently there has been a ban on the application of TBT based paints since 1st of January 2003, and such paints are mandated to be removed from commercial and recreational vessels from 1st of January 2008 (Yebra, Kiil et al. 2004).

In order to achieve the broad spectrum antifouling effect similar to TBT, most of the currently used AF paints incorporate copper in the form of cuprous oxide along with organometallic co-biocides. Copper release rates for some of the commercially used copper based coating are around 48.6 µg/cm²/d (Finnie 2006). As a result of booming global trade combined with increased ship sizes, the amount of copper released from such AF coatings is substantial and can cause adverse effects on the marine environment. Accumulation of leached copper can cause havoc in the marine ecosystem in ports around the world. Copper coated vessels can aid the transport and establishments of copper tolerant non-indigenous species to new habitats (Dafforn, Glasby et al. 2008).

Another class of AF coatings termed, non stick fouling release coatings, depend entirely on surface characteristics to release fouling (Olsen, Pedersen et al. 2007). Such coatings prevent biofouling by providing ultra-smooth, low friction surfaces on which
biofoulers have difficulties in settling. Widespread use of this technology is limited due to some drawbacks. Fouling release coatings require a minimum speed of 22 knots (kn) to completely remove attached biofouling, a minimum speed of 7 kn is needs to be attained to remove barnacles, 18 kn to remove weeds, while attaining speeds even above 30 kn are unable to remove the slime film. Thus the application of such coatings is restricted to high speed vessels. Fouling release coating are vulnerable to mechanical damage and tend to have shorter life time after which the mechanical properties of the coating deteriorate (Yebr, Kiil et al. 2004).

Current research efforts for designing an effective antifouling coating are focused on understating the biology of the fouling organisms. Interfering with the settlement and attachment mechanisms of the fouling organisms is being looked at as the most promising and environmentally benign option (Burgess, Boyd et al. 2003; Yebr, Kiil et al. 2004). Numerous studies and AF coating development efforts have focused on identifying and isolating secondary metabolites and bacterial extracts having AF properties (Maki, Rittschof et al. 1992; Burgess, Boyd et al. 2003).

Catecholamines, specifically adrenoreceptor agonists and antagonists have been shown to inhibit larval settlement in oyster larvae (Mollusca) (Coon, Bonar et al. 1985; Coon, Bonar et al. 1986; Coon and Bonar 1987), barnacle cyprid larvae (Arthropoda) (Yamamoto, Katsuhiyo Shimizu et al. 1999; Dahlström, Mårtensson et al. 2000; Dahms, Jin et al. 2004; Dahlström, Lindgren et al. 2005), Bugula neritina larvae (Bryozoa) and larvae of Hydroides elegans (Polychaeta) (Dahms, Jin et al. 2004) Due to its broad
ranging effects on inhibiting larval settlement, adrenoreceptor agonist noradrenaline was chosen for this study.

Exploration of surfaces by invertebrate larvae is an important step leading to attachment and metamorphosis to juveniles (Crisp 1974; Pawlik 1992; Hadfield 1998). Before settling, oyster veliger larvae and barnacle cypris larvae investigate surfaces using exploratory structures bearing an array of sensory cells (Clare 1995; Zimmer-Faust, Tamburri et al. 1996; Walters, Miron et al. 1999). The choice to attach and metamorphose is made upon receiving favorable cues. Despite the plethora of cues, there is a consensus amongst the literature that the choice to cement and metamorphose is only made after the larva has received the particular cue through its exploratory organs, such as the settlement disc on the 2nd and from the setae of 3rd and 4th antennular segments of barnacle cyprid and the foot of the molluscan veliger larvae (Crisp 1967; Clare, Freet et al. 1994; Clare 1995; Lagersson and Høeg 2002; Lagersson, Garm et al. 2003). It has also been shown that barnacle cyprids respond more favorably to settlement cues adsorbed on surfaces as opposed to settlement cues present in solution (Crisp and Meadows 1963; Dahlström, Mårtensson et al. 2000; Dahlström, Lindgren et al. 2005). This crucial act of exploration and sensing a surface can be used to engineer a fouling deterrent coating which can be an innovative strategy to prevent invertebrate larval fouling.

Noradrenaline (NA) is an agonist for the β-adrenergic receptor (β-AR), a G-protein coupled receptor (GPCR). GPCRs also known as the 7-transmembrane (7-TM) receptors and are present on the plasma membrane of the cells (Rasmussen, Choi et al.
Upon binding with the agonists, the receptors mediate cellular signaling through their cytoplasmic domains (Wolfe and Trejo 2007).

We hypothesize a scenario where signaling molecules like noradrenaline will be covalently immobilized to surfaces available for exploration to invertebrate larvae and would present ‘un-favorable’ cues deterring the larvae from settling on the surfaces. NA molecules covalently immobilized to a surface would need to be accessible to the sensory cells of the invertebrate larvae. Thus the objective of this study was to evaluate the accessibility of such immobilized noradrenaline molecules to adhering cells and their ability to effectively bind the β-AR and mediate downstream effects. To better understand the cell and immobilized NA interactions, immune cells or hemocytes of the Eastern Oyster *Crassostrea virginica* were chosen. Soluble NA has been demonstrated to mediate apoptosis in oyster hemocytes (Lacoste, Jalabert et al. 2001; Lacoste, Malham et al. 2001; Lacoste, Cueff et al. 2002) and the oysters constitute major invertebrate biofoulers.

**Materials and methods**

**Oyster Culture**

Adult Eastern Oyster *Crassostrea virginica* were purchased from Pemaquid Oyster Company Inc (Waldoboro, Maine). The oysters were acclimated in a 180 gallon (681 liter) tank at 18°C in artificial sea water at 31% salinity with saturating levels of dissolved oxygen. The animals were fed twice a week with Shellfish Diet 1800® (Reed
Mariculture Inc). To obtain hemocytes, the shell was notched with a cement saw to enable hemolymph extraction from the adductor muscle. A 22 gauge needle affixed to a disposable syringe was inserted into the adductor muscle and approximately 1 ml of hemolymph was withdrawn (Lacoste, Cueff et al. 2002).

**Engineering NA conjugated polymer surfaces**

NA-conjugated polymer surfaces were provided by Dr. Andrew Metter’s laboratory at Department of Chemical and Biomolecular Engineering at Clemson University. The process of engineering these polymer surfaces is briefly discussed here.

The bioactive surfaces were prepared on 1cmx1cm glass substrates first depositing a covalently attached monolayer of the iniferter SBDC (N,N(dimethylamino)dithiocarbamoylbenzyl(trimethoxy) silane) (de Boer, Simon et al. 2000; Rahane, Kilbey et al. 2005). The SBDC iniferter generates surface-bound reactive radicals on exposure to UV light. These surfaces were then covered with 75%v/v HEMA (2-hydroxyethyl methacrylate 99%) or MAA (methacrylic acid 99%) monomers solution and exposed to 365nm collimated UV light for 30-60 mins. This caused poly (HEMA) or poly (MAA) chains to be grafted from the surface. HEMA and MAA were selected as they provide -OH and -COOH reactive side groups respectively for covalently attaching Noradrenaline (NA). For covalent conjugation of NA, the carbonylimidazole (CDI) chemistry was selected. In the first step the polymer reactive groups were activated using
a CDI solution in anhydrous DMSO. To conjugate the primary amine group of NA to the reactive groups, polymers were incubated with a solution of NA in pH 8.0 buffer.

Definition of control surfaces

To assess the effect of NA-conjugated polymer surfaces on adhering hemocytes a variety of control surfaces were tested in parallel to NA-conjugated polymers. Control surfaces included glass coverslips, MAA and HEMA polymer surfaces without NA conjugation and from here on will be referred to as control surfaces. As hemocyte response to all of the control surfaces was similar, data presented here are representative images from either of the control surfaces.

Viability assay

Oyster hemocytes were obtained as described earlier. Approximately 500 μl of hemolymph was aliquoted on control surfaces, NA-HEMA and NA-MAA polymer surfaces. Hemocytes were incubated at 18°C for 45 minutes. To remove unattached cells, substrates were washed twice for three minutes each with molluscan phosphate buffer saline (PBS) (PBS osmolality was matched to the hemolymph). Each substrate was then placed in 60x15mm petri dishes (Fisher Scientific) containing molluscan PBS at 18°C. Ten micromolar solution of Calcein-AM (Invitrogen, Carlsbad, CA, cat#C3099) in molluscan PBS was added to substrates and incubated for 20 minutes in dark. Substrates
were then washed twice for five minutes each with molluscan PBS to remove excess dye. Calcein-AM is a fluorophore coupled with an aceto-methoxy ester. Living cells possessing estarases enzymes take in the dye and cleave the AM moiety of the dye rendering it fluorescent.

Hemocytes were counterstained with a 300 nM solution of 4′,6-diamidino-2-phenylindole dihydrochloride or DAPI (Invitrogen cat#1306) for five minutes. Substrates were washed twice for five minutes each with molluscan PBS to remove excess DAPI. Observations of samples were performed using Carl Zeiss Axiovert-135 inverted microscope (Carl Zeiss Inc. Thornwood, NY) with appropriate filter sets for Calcein-AM and DAPI with a 40X oil immersion objective, using Carl Zeiss Axiocam MRC-5 camera.

Cytoskeletal assay

Oyster hemocytes were obtained as described earlier and incubated on the control, NA-MAA and NA-HEMA surfaces. Hemocytes were allowed to adhere and settle on substrates at 18°C for 45 minutes. Samples were prepared as described previously. Cells were then fixed on substrates with 4% paraformaldehyde made up in PBS for 30 minutes followed by three (PBS) washes for five minutes each to remove unreacted paraformaldehyde. To permeabilize the cells, substrates were incubated in a 0.1% solution of Triton-X 100 for five minutes followed by two washes of three minutes each. Substrates were then incubated in a 50 μM solution of FITC-Phalloidin (Sigma-Aldrich,
cat#P5282) for 30 minutes, followed by two washes of 5 minutes each to remove excess phalloidin. Hemocytes were then counterstained with DAPI and fluorescence microscopy was performed as previously described.

Assessing the ability of NA-conjugated polymer surface to induce apoptosis

Samples were prepared as described previously. Samples were analyzed using Vybrant Apoptosis Assay Kit #11 (Invitrogen, cat#V35116). Substrates were incubated with the mitochondrial dye MitoTracker Red followed by a brief wash, cells were incubated with annexin binding buffer and Alexa Fluor 488 Annexin-V for 30 min. Fluorescence microscopy was performed as described previously.

Scanning electron microscopy studies

Hemocytes were incubated on control, NA-MAA and NA-HEMA polymer surfaces as previously described. Primary fixation of the samples was accomplished using a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde (Electron Microscopy Sciences, EMS) made up in 100mM sodium cacodylate buffer (EMS) for 30 minutes at 4°C. Substrates were then washed 3 times for 10 minutes each. Cells were then post fixed for twenty minutes using a 1% solution of osmium tetroxide (EMS) made up in sodium cacodylate buffer. Following post fixation, samples were washed with deionized water 3 times for 10 minutes each. To dehydrate the cells, samples were taken through a graded
ethanol series of 25%, 50%, 75%, 80%, 90%, 100%. In the graded series, dehydrations between 50%-100% ethanol were carried out at 0°C to minimize extraction. Samples in 100% ethanol were transferred to a chamber of critical point dryer (CPD). Substrates were subjected to 3 washes of liquid CO₂ for 5 minutes each, to remove ethanol from the sample; CPD was carried out at 1072 psi at 31°C. After CPD substrates were mounted on aluminum stubs using double sided carbon tape, and sputter coated with Denton sputter coater with a gold-palladium target. Samples were imaged using Hitachi Field Emission-S4800 and Hitachi-S3400 scanning electron microscopes.

Results

NA-conjugated polymer surfaces adversely affect the cytoskeleton of adhering hemocytes

Hemocytes adhering to the control surfaces label positive for calcein-AM and fail to label for DAPI (Fig-1A), indicating that polymer surfaces alone do not exert any deleterious effects on the adhering cells. Hemocytes adhering to NA-MAA or NA-HEMA surfaces failed to exhibit any fluorescence from calcein-AM labeling and label positive for DAPI (data not shown), indicating that cell viability is compromised upon adhering to these surfaces.

Polymer surfaces are known to exert an effect on the cytoskeletal structure of the adhering cells (Shunji Nagahara 1996; Yamato, Okuhara et al. 1999; Shin-Ichi Sawada, Iwasaki et al. 2006). Effect of NA-conjugated polymer surface was evaluated by
phalloidin labeling the actin filaments of the adhering hemocytes. Cells adhering to the control surfaces show a diffused pattern of actin filaments and an absence of stress filaments (Fig.1B). Hemocytes adhering to NA-MAA surfaces displayed stressed actin filaments (Fig.1C-D). Abnormal localization of actin and disintegrating cell membranes were also detected in adhering hemocytes (Fig. 1C-D arrows). Cells adhering to NA-HEMA surface can be seen to have undergone advanced disintegration (Fig.1E-F). Degradation of cytoskeleton appeared to have lead to loss of nuclei from adhering hemocytes. Enucleated hemocytes with extracellular nuclei can be seen in Fig.1F. Control surfaces alone do not exert any adverse effects on the cytoskeleton of the adhering cells. NA-conjugated HEMA/MAA polymer surfaces induce cytoskeletal degradation in adhering hemocytes.
**Figure-1:** Effects of NA-conjugated polymer surface on the cytoskeletal structure and viability of adhering hemocytes. **A)** Fluorescent micrograph of hemocytes incubated on control surfaces and labeled with calcein-AM and DAPI. Green fluorescence indicates adhering cells are healthy and viable. **B)** Fluorescent micrograph of hemocytes incubated on control surfaces labeled with FITC-phalloidin labeling F-actin. There is no evidence of the any stress or abnormalities on the cytoskeletal structure of the adhering hemocytes. **C-D)** Fluorescent micrograph of hemocytes incubated on NA-MAA polymer surfaces labeled with FITC-phalloidin and DAPI, labeling F-actin and nuclei respectively. Hemocytes adhering to NA-MAA polymer show abnormal and stressed cytoskeleton (red arrowheads). Disintegrating cells and abnormal accumulation of actin is clearly seen (arrows). **E)** Fluorescent micrograph of hemocytes incubated on NA-HEMA polymer surfaces labeled with FITC-phalloidin and DAPI. Pronounced cytoskeletal disintegration is evident with cells displaying abnormal morphologies (arrows). **F)** Hemocytes incubated on NA-HEMA polymer surfaces labeled with FITC phalloidin and DAPI. Enucleated hemocytes with disintegrating cytoskeletons can be seen (arrows). As a consequence of cytoskeletal deterioration nuclei are released from the hemocytes and can be observed extracellularly (red arrowheads). Scale bars: A=15 μm. B=10 μm. C, D =12 μm. E,F= 15 μm
NA-conjugated polymer surfaces induce apoptosis in adhering cells

Hemocytes incubated on the control surfaces display intense staining with MitoTracker Red indicating that the cells are viable and healthy. These cells fail to label for Annexin-V indicating an absence of apoptosis (Fig. 2A). Hemocytes incubated on NA-MAA and NA-HEMA polymer surfaces fail label for MitoTracker Red but label positive for Annexin-V, indicating that the cells undergo apoptosis as a consequence of interaction with NA conjugated polymer surfaces (Fig. 2B).
**Figure-2:** Interaction between hemocytes and NA-conjugated polymer surfaces analyzed using Annexin-V apoptosis assay. **A)** Hemocytes incubated on control surfaces labeled positive for MitoTracker red indicating viable and healthy cells. **B)** Hemocytes incubated on NA-conjugated polymer surfaces labeled positive for Annexin-V indicating that cells are undergoing apoptosis. This is a representative micrograph from NA-MAA polymer surface, cell show similar pattern of labeling on NA-HEMA polymer surface. Scale bar: A, B=10 μm

Cytoskeletal destruction in hemocytes adhering to NA-conjugated polymer surfaces observed by scanning electron microscopy

SEM analysis revealed that hemocytes adhering to control surfaces exhibit a normal morphology. Control hemocytes show intact cell membranes, cells can be seen spreading over the surface (Fig. 3A). Hemocytes incubated on NA-MAA surfaces exhibit abnormal morphology, the cell membranes can be seen disintegrating or blebbing off towards the periphery of the cells (Fig. 3B-D). Cells incubated on NA-HEMA exhibit similar effects as those incubated on NA-MAA surface. Disintegrating cell membranes resembling apoptotic bodies are clearly visible (Fig.3C-E). An indicator that NA-conjugated surfaces induce apoptosis in adhering cells, two adjacent hemocytes adhering to NA-MAA surface, one cell can be seen disintegrating while the other remains intact (Fig.3F).
Figure-3: Interaction between hemocytes and NA-conjugated polymer surfaces analyzed using SEM. A) Hemocytes adhering to control surfaces display normal morphology and uncompromised cell membranes. B-D) Hemocytes incubated on the NA-MAA polymer surfaces exhibit compromised cell membranes. The area inset square box is observed under higher magnification in D. Disintegrated cell membranes of the two adjacent cells are clearly visible. C-E) Hemocytes incubated on NA-HEMA polymer surfaces show pronounced membrane deterioration. A hemocyte with a disintegrated morphology is seen (arrows). Area inset box is observed under higher magnification in E, small cytoplasmic fragments (blebs) can be seen around the periphery of the cell. F) Adjacent hemocytes adhering to NA-HEMA polymer surface, one of the hemocytes is disintegrating while the adjoin cell appears to be intact.
Discussion

Polymer surfaces, by virtue of their physical characteristics exert a variety of effects on adhering cells, from inhibiting cell attachment to affecting cytoskeletal structure (Shunji Nagahara 1996; Yamato, Okuhara et al. 1999; Shin-Ichi Sawada, Iwasaki et al. 2006). MAA and HEMA polymers in the absence of NA conjugation did not appear to compromise cell viability. This was demonstrated by viability staining using calcein-AM and DAPI (Fig. 1A). Cells adhering to NA-MAA and NA-HEMA polymer surfaces failed to label for calcein-AM and labeled positive for DAPI indicating that viability of cells adhering to NA conjugated polymers was compromised.

Control surfaces also failed to exert any deleterious effects on the cytoskeleton of adhering hemocytes. As demonstrated by phalloidin staining (Fig.1B), well preserved cell morphologies and intact plasma membranes were observed. Cells incubated on the NA-MAA and NA-HEMA polymer surfaces displayed abnormal cell morphologies and deteriorating plasma membranes (Fig. 1 C-F). Cytoskeletal degradation along with abnormal accumulation of cytoskeleton was also observed (Fig. 1D, E, F). Scanning electron microscopy (SEM) analysis of cells interacting with NA-conjugated polymers corroborated results obtained from phalloidin staining. Plasma membrane deterioration (Fig.3B-F) in the hemocytes adhering to NA-conjugated polymer surfaces appeared strikingly similar to membrane blebbing occurring during the late stages of apoptosis (Coleman, Sahai et al. 2001; Lacoste, Cueff et al. 2002). Hemocytes adhering to control
surfaces did not show any signs of membrane blebbing or membrane deterioration (Fig. 3A).

Evidence from viability and phalloidin staining indicated the possibility of cells undergoing apoptosis after interacting with NA-conjugated polymer surfaces. Chromatin condensation, degeneration of mitochondrial membrane potential and exposure of phosphatidylserine at the plasma membrane are hallmarks of the apoptosis pathway (Gourlay and Ayscough 2005). Hemocytes adhering to NA-conjugated polymer surfaces labeled positive for annexin-V and failed to label for the mitochondrial dye (Fig. 2B). Annexin-V labeling indicated translocation of phosphatidylserine to the outer leaflet of the plasma membrane along with failure to label for the mitochondria confirmed the progression of apoptosis in hemocytes.

Hemocytes adhering to MAA, HEMA and other control surfaces failed to label for annexin-V while labeling positive for the mitochondrial dye, indicating absence of apoptosis. As MAA, HEMA and other control surface failed to induce apoptosis in hemocytes; the presence of NA molecules on polymer surfaces can be attributed for inducing apoptosis. Although the pathway through which apoptosis is induced remains to be investigated, following pathways are hypothesized through which NA conjugated polymer surfaces exert these effects.

β-ARs have been demonstrated to induce apoptosis through Lck, a Src-family tyrosine kinase which is independent of protein kinase-A (PKA) (Gu, Ma et al. 2000; Ma and Huang 2002). Perhaps the covalently conjugated NA molecules induce apoptosis in the hemocytes via Lck pathway. β-AR stimulation by agonist also leads to stimulation of
L-type Ca\(^{2+}\) channels (Juming, Joseph et al. 2001; Xiao 2001), influx and overload of Ca\(^{2+}\) as a result of L-type Ca\(^{2+}\) channel stimulation has been demonstrated to cause apoptosis in cells (Juntti-Berggren, Larsson et al. 1993). Thus an incessant stimulation of β-AR from NA-conjugated polymers could lead to unregulated Ca\(^{2+}\) influxes triggering apoptosis in adhering cells.

“Desensitization and receptor trafficking are the predominant mechanisms that control G Protein Coupled Receptor (GPCR) signaling. Activated GPCRs are rapidly desensitized by phosphorylation and arrestin binding that promotes receptor uncoupling from G proteins. G protein-coupled receptor internalization ensues within minutes and removes activated receptor from G proteins and signaling effectors at the plasma membrane. Once internalized, some GPCRs may continue to signal from endosomes, until agonist eventually dissociates from receptors or receptor signaling is shut off. G protein-coupled receptors are then dephosphorylated and recycled back to the cell surface” (Wolfe and Trejo 2007). Whether the process of receptor internalization is hindered by covalently conjugated NA molecules remains to be investigated. It is tempting to speculate that hindrance in agonist internalization may result in unregulated signaling by the agonist bound receptor, leading to apoptosis and array of other deleterious effects on the adhering cells.

Two possible scenarios could also be speculated to explain the cytoskeletal deterioration and enucleation observed in hemocytes adhering to NA-conjugated polymer surfaces (Fig. 4C-F). As a result of apoptosis, actin and other elements of cytoskeleton are cleaved by caspases activity (Gourlay and Ayscough 2005), apoptosis could thus be a
major cause of cytoskeletal deterioration. It has also been shown that actin cytoskeleton can function as a regulator for reactive oxygen species (ROS). Release ROS from the mitochondrion, and actin stabilization can induce cell death (Simon, Haj-Yehia et al. 2000; Gourlay and Ayscough 2005). Thus the cytoskeletal deterioration could not only be a consequence of apoptosis but may also promote apoptosis through a feedback loop.

Another pathway can be hypothesized to explain the observed cytoskeletal deterioration. The G protein-coupled receptor kinase 2 (GRK2) is a serine/threonine kinase that phosphorylates and desensitizes agonist-occupied GPCRs (Pitcher, Hall et al. 1998). After recruitment to the phosphorylated receptor, activated Src phosphorylates and activates GRK2 (Ma and Huang 2002). Recruitment and activation of GRK2 to the plasma membrane leads to GRK2-tubulin complex formation and tubulin phosphorylation, GRK2 thus acts as a signal transducer which mediates the effects of GPCR activation on the cytoskeleton (Pitcher, Hall et al. 1998). Since NA molecules are covalently conjugated and cannot be internalized, β-AR stimulation could lead to unregulated GRK2 mediated tubulin phosphorylation at the plasma membrane, causing detrimental effects on the cytoskeleton and in turn cell viability.

From the results it can be inferred that although covalently conjugated to polymer surfaces, NA molecules are able to induce a myriad of effects in adhering hemocytes. These encouraging although preliminary results, warrant further research in characterization of these surfaces and the cellular effects mediated by them.

Further research is also warranted to understand effect of NA-conjugated polymer surfaces on invertebrate larval settlement. New insights will be gained by understanding
the interactions between NA-conjugated polymer surfaces and larval exploratory organs. This research will help in the development of fouling deterrent strategies which will specifically target fouling mechanisms in invertebrate larvae.

β-AR belongs to the GPCR family which is the largest and most diverse family of signaling receptors encoded in the mammalian genome comprising of ~900 members (Wolfe and Trejo 2007). GPCR transduce signals of diverse extracellular stimuli such as photons, ions, biogenic amines, lipids, bile acids, peptides, proteases etc. The diversity of these receptors is evident by the role of GPCR signaling in disparate physiological responses such as vision, olfaction, cardiac function, gustation and neurotransmission (Wolfe and Trejo 2007). Nearly half the drugs in use currently target GPCRs either directly or indirectly thus importance of GPCR signaling pathways for therapeutic development is evident (Wolfe and Trejo 2007). GPCR agonist bound polymers, such as NA-MAA and NA-HEMA polymers could open up new avenues for understanding GPCR signaling from a new perspective.

In experimental approaches, single molecule manipulations could be used to study the interactions between covalently linked agonists and GPCR bearing cells (Zhou, Nakatani et al. 2007). GPCR ligands conjugated to quantum dots (Zhou, Nakatani et al. 2007) and nanocapsules (D. Silvano, Kro et al. 2002) could be used to study receptor dynamics using advance microscopy techniques such as fluorescence resonance energy transfer (FRET) (Petty 2007) and optical trapping (Keir and Steven 2004; Cojoc, Difato et al. 2007). In a FRET pair experiment, polymer conjugated NA could be used in conjunction with fluorescently tagged GPCR bearing cells (Petty 2007). FRET
experiments could shed light on possibility of covalently bound agonist impeding receptor endocytosis and prolonging agonist-receptor interaction time in a quantifiable manner.

Other single molecule techniques such as TIRF (total internal reflection microscopy) could be combined with FRET to visualize only the vicinity of the cell membrane, eliminating the noise from rest of cell volume (Axelrod 2001; Schneckenburger 2005). Interaction between covalently bound agonist and receptor can also be used to gain valuable insights about the mechanical kinetics of endocytosis and fluidity of the cell membrane. An experiment can be conceived where GPCR-agonists are conjugated to polymer beads which are manipulated with laser tweezers and allowed to interact with GPCR bearing cells (Wei, Tromberg et al. 1999). In a ‘tug of war’ scenario the agonist bearing beads could be pulled back and forth to quantitatively determine the force exerted by clathrin, arrestin anddynamin mediated endocytosis on the agonist bound receptor. This will also yield mechanistic information about the fluidity of the cell membrane. This information could be of potential use in various fields, from designing microfluidic systems, tissue engineering and designing bioreactors, to understanding parasite-host cell interactions and virus-host cell interaction etc.

β-AR antagonist pharmacologically referred to as β-blockers are of great therapeutic importance in treatments of hypertension, angina and acute coronary syndrome, supraventricular and ventricular arrhythmias and congestive heart failure (Antelava, Antelava et al. 2006; Wiysonge, Bradley et al. 2007). As agonist conjugated polymers affect adhering hemocytes, β-blocker-conjugated polymers might be able to
exert remedial effects by down regulating β-AR driven signaling pathways. Thus further research is warranted in understanding the effect of β-blocker-conjugated polymers on adhering cells and may hold a promise of discovering novel therapies for cardiovascular ailments.

The finding that covalently conjugated GPCR agonists such as NA are able to mediate effects on adhering cells is a novel and holds the possibility of revolutionizing the fields of biofouling, bioengineering to drug discovery.
CHAPTER FIVE

SUMMARY

Noradrenaline stimulates apoptosis in approximately 50% of the hemocyte population (Chapter 2), perhaps indicating the presence of a paracrine system of neuroendocrine control in oysters. This ‘stress response’ has been described by Lacoste et al. (2001) as a series of orchestrated physiological reactions, increasing an organism capacity to maintain homeostasis in the presence of threatening agents. Various assays discussed in chapter 2 could be modified and used to study the effects of the antifouling compounds on invertebrate larvae. Studying the effects of antifouling compounds at a cellular and organismal level would lead to better understanding of the mechanisms through which these compounds mediate their effects. Holistic comprehension of such mechanisms would lead to the development of environmentally responsible antifouling strategies and would prevent environmental catastrophes such as the one caused by TBT paints.

The proteomics techniques that were used to identify the β-AR present on oyster hemocytes are also useful for identifying proteins involved in the larval settlement process. As more oyster and barnacle genomic information becomes available, discovery bioinformatics will help identify gene sequences coding the proteins identified by mass spectral analysis. Functional studies using RNA interference (RNAi) (Hannon 2002) or the gene knockout technology (Galli-Taliadoros, Sedgwick et al. 1995; Mak, Penninger et al. 2001) could be then carried out to further indentify and discern the various
mechanisms involved in marine invertebrate larval settlement and metamorphosis. Similar approaches to NA treated oyster and barnacle larvae could be applied to other marine invertebrate larvae for understanding various signal transduction cascades initiated and mediated by neuromodulators. From this and previous studies it is evident that NA affects settling behavior in both oyster and barnacle larvae, yet the exact mode of its action remains to be understood. A multifaceted approach which includes invertebrate cell biology combined with genomics, proteomics and advanced imaging techniques could elucidate various aspects of NA mediated inhibition of larval settlement.

Gregariousness is a hallmark of many sessile marine invertebrates including oysters and barnacles. This settling behavior is crucial to larval survival and is thought to be a resultant of genetic changes which enable the larvae to become competent and metamorphose (Coon, Fitt et al. 1990). NA treatment which uncouples settling behavior from metamorphosis could be used in conjunction with genomic and proteomic approaches to tease out key genes and signal transduction pathways involved in this process. Such an approach could shed new light on many fundamental aspects of marine invertebrate development. Thus such a ‘drug discovery’ approach could also lead to the development of efficient antifouling compounds which target specific and well understood mechanism with little or no impact on non-target organisms or the environment.

Covalently conjugated NA molecules induced apoptosis in the adhering hemocytes of *C. virginica* (Chapter 4). The cytoskeleton of these cells shows severe deterioration, possibly as a consequence of apoptosis. The exact mechanism through
which NA-conjugated surfaces induce apoptosis is unknown and requires further investigation. It is hypothesized that incessant stimulation of β-AR by covalently conjugated NA molecules could lead to induction of apoptosis.

To date an overwhelming majority of antifouling coatings have included biocides which leach out of the coatings to mediate their effects. Advances in polymer technology have led to development of ‘non-stick’ or fouling release coatings which have low friction surfaces and release the accumulated fouling as the vessel picks up speed (reviewed in chapter 1). Yet such coatings lack a specific deterrence strategy targeted towards invertebrate larvae.

Advances in polymer chemistry have also lead to the capability of covalently conjugating specific molecules to polymer surfaces. Perception of surface immobilized chemical stimuli by both oyster and barnacle larvae have been adequately demonstrated (Crisp and Meadows 1962; Crisp and Meadows 1963; Crisp 1967; Crisp 1974). Surface adsorbed adrenoreceptor molecules have also been shown to inhibit settlement of B. improvisus larvae (Dahlström, Mårtensson et al. 2000). Further research on the effects of NA conjugated polymer surface on the settling behavior of oyster and barnacle larvae is needed to ascertain if this is a viable fouling deterrence strategy.

GPCRs constitute largest family of receptors with ~900 known members, ~50% of all the drugs in use currently target GPCRs in one way or the other. As the crystal structure of β-adrenergic receptor has been recently elucidated (Rasmussen, Choi et al. 2007; Rosenbaum, Cherezov et al. 2007), understanding the effects of a surface conjugated agonists or antagonists on receptor dynamics will yield valuable information
on β-adrenergic receptor mediated signal transduction. Such studies could prove to be invaluable in therapeutic development. As the β-AR structure is known, it is possible to design synthetic agonists using computer modeling (Ivanov, Baskin et al. 2005; Zuo, Chen et al. 2007; Kimura, Tebben et al. 2008) making it theoretically possible to design GPCR agonists and antagonists possessing properties which make them amenable to large scale production and incorporation into various polymer systems.

The fouling deterrence strategies discussed in this dissertation could perhaps be explored as an alternative to biocide releasing coatings. Surface exploration by invertebrate larvae could be taken advantage of while designing a new generation of antifouling coatings. Such strategies could be incorporated into various polymer systems such as the fouling release coatings, super hydrophobic coatings (Genzer and Efimenko 2006) etc. to form an antifouling coating system which targets a broad spectrum of fouling organisms.

Biofouling has been combated throughout maritime history. We have started understanding and appreciating the complexities of fouling organisms in our recent past. Marine fouling organisms are numerous and diverse thus finding a single antifouling solution effective against all them is a Herculean task. The resolution to this phenomenon will come only after understanding the incredible capabilities endowed to these organisms through millions of years of evolution and effectively countering them in manner which is safe, effective and environmentally responsible.
WORK CITED


