Ability of Invertebrates and Algae to Colonize Biocidal Nanoparticles

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Undersurfaces of ship hulls are subject to "fouling"/"biofouling." Historically, copper sheets bolted to wooden warships[1] and current military and commercial anti-biofouling "bottom paint" rely upon the slow release/leaching of copper ions (cuprous ion, Cu^+) despite its disadvantages: 1) Cu^+ is not quite toxic enough to totally prevent biofouling, and 2) most Cu^+ eventually leaches into the seawater, rendering the paint ineffective in anti-fouling and contaminating surrounding waters with Cu^+. These shortcomings require that ships be periodically dry-docked, stripped, and re-painted, maintenance procedures costly in time, personnel, and materials. Because Tributyl-Tin (TBT) has been linked with the disappearance of a variety of organisms from harbors, especially mollusks, such as whelks and oysters, use of TBT Self-Polishing Co-Polymer paint was globally prohibited in 2003, and must be completely eliminated by 2008[1]. Alternative methods for inhibiting biofouling on hulls therefore are actively sought. Using modern techniques for fabricating nanoparticles[6] composed of chemical compounds hypothesized to possess anti-fouling properties[3] and not to release their component chemicals by leaching, we prepared nanoparticle-based substrata composed of four candidate formulations whose initial tests in open seawater produced promising anti-fouling results, as presented below. These studies also provide basic, non-applied data on expression of "stress-response" genes in a variety of organisms in response to contact with these unusual surfaces[10].

The goal of our research is to devise nanoparticle-loaded matrices that a) will be so non-adhesive and toxic that any and all living organisms will be either unable to adhere and/or repelled (though not necessarily killed) even by simple contact/adhesion/attachment to the nanoparticle-loaded surface, and b) yet will not release/leach toxic products into the seawater of anchorage harbors. Nanoparticles provide extraordinarily large total surface areas per weight of compound[8]. The chemical and biological properties of a compound when fabricated in nanoparticle form often bear little resemblance to those of the same compound if fabricated in standard bulk configuration[5]. In addition, a very great variety of active, functional chemical groups can be coupled covalently to nanoparticle surfaces. During 2005, four types of functional groups were attached to nanoparticles by the KJK lab at Kansas State University: 1. Cr/SiO_2, 2. n-hexadecyltrimethylammonium bromide ($CH_3(CH_2)_{15}N(CH_3)_3Br$); 3. Fluorine; and 4. MgO_2. The coated nanoparticles were immobilized in paint-like polyurethane matrix and applied to glass slides. Anti-biofouling activity of these matrices was compared to that of (a) simple polyurethane matrix, (b) surfaces coated with commercially available copper bottom paint, and (c) uncoated glass slide surfaces. During June and July, sets of slides were held in slide boxes whose sides had been sawed-out and allowed to incubate while suspended in open seawater. At weekly intervals, sets of both control and experimental slides were removed for analysis, either immediately (in fresh, wet condition) or after being allowed to air-dry overnight. Growth of organisms on the slide surfaces was then documented qualitatively by digital photography, followed by collection of all material (substrata, as well as any attached organisms) by rapid scraping and transfer into microfuge tubes containing solutions that prevent degradation of RNA or DNA.

Growth of organisms on 1. Cr/SiO_2/Polyurethane was totally inhibited, whereas growth occurred on both polyurethane control surfaces and glass after one week. Growth of organisms on 1. Cr/SiO_2/Polyurethane and on 2. n-hexadecyltrimethylammonium bromide ($CH_3(CH_2)_{15}N(CH_3)_3Br$)/ Polyurethane were highly inhibited compared with growth on glass after 4 and 6 weeks. Growth of

organisms on 3. fluorinated polyurethane, was not inhibited compared to growth on glass after 1.5, 2.5 or 4 weeks. Growth on 4. MgO_2/Polyurethane was inhibited compared with that on both polyurethane film and on glass after 1.5 weeks. Comparisons 1 & 2 were made vs. growth on glass at the longer time intervals because during incubation in seawater, the control polyurethane substrata detached from the glass and could not be evaluated, whereas the nanoparticle-loaded matrices tended to remain adherent to the glass during the 7 weeks of incubation in seawater. Organisms were attached to the surface of slides coated with copper bottom paint after 2.5 weeks, but they may have been dead, because they lacked green color compared with the dark-green growth of organisms on the glass control substratum. Such green-deficient material did not appear to attach to nanoparticle-loaded formulations #s 1 and 2 above, however, suggesting that organisms may not even attach at all to nanoparticle #1- and #2-coated surfaces. Further study will seek a simple method for treating the glass surfaces to prevent polyurethane and nanoparticle-loaded polyurethane matrices from detaching during incubation in seawater, such as treatment of surfaces with a "toughening tie layer"[2].

We used three molecular techniques for assessing growth and viability of a wide diversity of organisms. All organisms from a surface were removed by mechanical scraping, followed by either: a) isolation and quantification of total DNA by simple ultraviolet spectrometry, b) isolation of DNA, followed by PCR (polymerase chain reaction) with degenerate primers for a highly conserved region of mitochondrial CO1 (cytochrome oxidase), and sequencing of CO1 fragments obtained to reveal the diversity of species that adhere to different compositions of nanoparticle-loaded substrata, or c) isolation of total RNA, followed by RT-PCR (reverse-transcriptase-polymerase chain reaction) with degenerate primers for CO1 to determine whether the adherent organisms were alive (lack of CO1 expression, despite visual evidence of organism attachment, would suggest that organisms attached, but then died). Mitochondrial CO1 is often used to determine species differences in groups of organisms[11,12]. Once individual species of fouling organisms have been identified, the relative abundance of these species on various different nanoparticle surfaces can be quantified using species-specific primers and Real Time PCR. During summer 2005, when DNA-based PCR assays or RNA-based RT-PCR assays were performed using degenerate primers for CO1 and reduced hybridization temperatures, PCR bands of expected size (by gel electrophoresis) were obtained. We must confirm by cloning and sequencing the CO1 fragments we have obtained that they are indeed CO1. Material from which to harvest DNA and whole cell-RNA from experimental and control slides coated in nanoparticle # 1-4 substrata and control polyurethane-coated or glass slides has been collected and awaits further analysis as described above.

Although the goal of this research is initially applied (devise anti-biofouling surfaces more effective than copper bottom paint), it is expected to serendipitously reveal some unexpected properties of organisms that will be of great basic research interest. Cells are sensitive even to physically touching nanoparticles[9] and other surfaces, reacting by rapid changes in gene expression[4]. For example, it should be possible to compare the relative toxicity of various nanoparticle-loaded matrices by comparing their abilities to elicit expression of "heat shock/chaperone" genes or "oxidative stress" genes in any organisms that adhere to them[7,10].

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