Novel application of imaging surface plasmon resonance for *in situ* studies of the surface exploration of marine organisms

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The surface interactions of exploring cyprids of the barnacle *Semibalanus balanoides* were studied *in situ* using imaging surface plasmon resonance. It was demonstrated how the deposition of a proteinaceous adhesive could be followed in real time as the cyprids explored and temporarily attached to a surface. Furthermore, the amount of protein left on the surface when the cyprids moved on could be quantified. Clear differences were demonstrated between an oligo(ethyleneglycol) coated surface and a bare gold substrate. It is anticipated that this technique will be a valuable tool in the development of novel surface chemistries that can prevent biofouling. © *2009 American Vacuum Society.* [DOI: 10.1116/1.3274060]

Marine biofouling is an international problem of great commercial and environmental significance. Ship hulls and other submarine structures are rapidly colonized by fouling organisms such as bacteria, algae, and sessile invertebrates. This makes the structures more prone to damage/corrosion and causes drag, which leads to increased fuel consumption, elevated shipping costs, and increased CO_2 emissions.[1] Barnacles are a significant component of hard fouling assemblages worldwide and there is considerable interest in the development of coatings that preclude their attachment and growth.[2]

The settling larvae of barnacles are known as cyprids. Prior to settlement, the cyprid explores the substrate, sensing the surface chemical and physical characteristics and ultimately selecting a surface that is deemed appropriate for growth and survival to adulthood. Exploration is facilitated by a temporary adhesion mechanism involving a proteinaceous material secreted from the end of each of the cyprid's two antennules. This system of temporary attachment, exploration, and surface sensing is common to many of the settling stages of sessile marine organisms.[3] It would therefore be useful to learn more about the adhesion processes involved, making intervention at an early stage in the fouling process a possibility. In the case of cyprids, observation of the deposition of proteinaceous "footprints" may provide such insights. It may be assumed that the frequency with which the secreted protein sticks to the surface rather than to the antennule provides a proxy indication of the strength of the protein-surface bond. Thus, with knowledge of the specific surface chemistry, this approach could also provide an insight into the chemistry and thermodynamics of adhesion.

The following text outlines the novel application of imaging surface plasmon resonance (iSPR) for evaluating putatively fouling-resistant surfaces. This work may represent the first adequate, rapid, and straightforward method for observing cyprid footprint deposition *in situ*. In this case, wild-caught cyprids of the boreoarctic barnacle *Semibalanus balanoides* were used (see the inset in Fig. 1).

Traditionally, methods such as scanning electron microscopy (SEM) and scanning probe microscopy (SPM) have been employed in this kind of study.[4] Neither of these techniques, however, allow for real time observation of the exploratory behavior of the organisms *in situ*. SEM and SPM are also less suitable for evaluation of larger surface regions. iSPR is an imaging optical transducer technique that measures the thickness or refractive index of thin adsorbed dielectric layers.[5] Because it is a label-free technique, capable of yielding a two-dimensional quantitative information map over the sensing surface, iSPR is widely employed in biosensors and for biomolecular interaction analysis.[6] The extreme surface sensitivity of SPR has also found applications in the visualization of cell/substrate contact[7] as well as for the study of cell adhesion and proliferation.[8,9]

Surface plasmons may be described as quantized surface-bound electromagnetic waves confined to and propagating along the interface of a metal and a dielectric. Surface plasmons can be resonantly excited by visible light in a setup known as the Kretschmann configuration wherein light, incident through a prism, is reflected from a thin metal film.[10] At resonance, attained for a specific angle of incidence by varying the wavelength, the incident light is coupled into surface plasmon modes. Reflectivity curves, wherein the position of the minima is proportional to the refractive index in the im-

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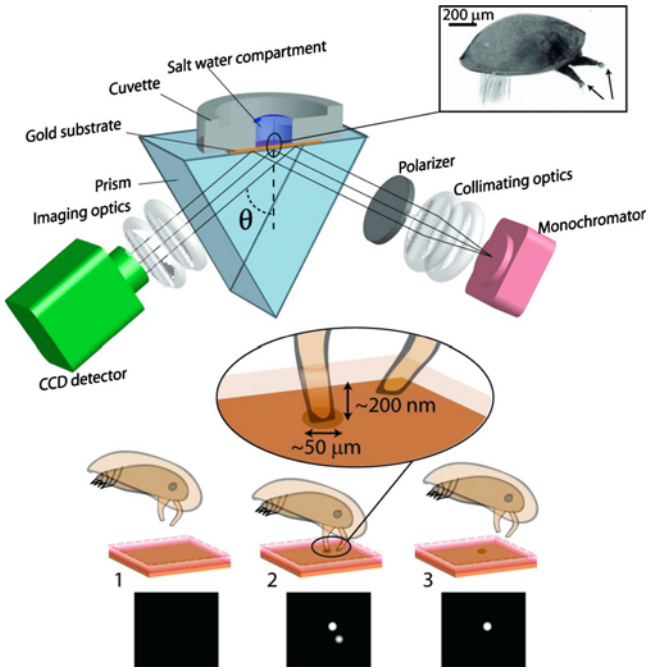

FIG. 1. (Color online) Cartoon of the imaging SPR experimental setup outlining the optical components and the cuvette. The inset shows the *Semibalanus balanoides* cypris larva and the arrows indicate the antennules. When the cyprids' attachment organs approach the surface, a bright spot appears in the SPR image. If adhesive material is released, the bright spot will remain as a footprint in the image when the cyprid moves on. The magnified view illustrates schematically the SPR probe depth of a few hundreds of nanometers. The diameter of the cyprid's attachment disks is about 50 μm.

mediate vicinity of the metal film, can be acquired by scanning the wavelength. Refractive index changes can also be sensed by measuring the shift in intensity at a fixed angle of incidence and wavelength. There is a vast literature on surface plasmons;[11] therefore we only briefly consider the characteristics that have important implications for the performance of the application described herein. The electric field associated with a surface plasmon bound at the interface (x,y plane) between two semi-infinite media is

$$\vec{E} = \vec{E}_0 e^{i(\vec{k}_x x - \omega t \pm (\vec{k}_{z,j} z))} \quad (j = 1,2). \tag{1}$$

The dispersion relations along the x and z directions are given from Maxwell's equations as

$$\vec{k}_x = \frac{\omega}{c}\sqrt{\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2}} \tag{2}$$

and

$$\vec{k}_{z,j} = \sqrt{\frac{\omega^2 \varepsilon_j}{c^2} - \vec{k}_x^2}, \tag{3}$$

where in ε_1, ε_2 denotes the dielectric functions of the metal and dielectric, respectively. Since ε_1 is complex while ε_2 is normally real, the surface plasmon wavevector $\vec{k}_x$ will have an imaginary part. The surface plasmon is, therefore, a damped wave and has a finite propagation length, L_x, in the x direction. Over this distance, the energy of the surface plasmon is dissipated as heat in the metal. Furthermore, the lack of propagating waves in the metal or dielectric requires that $\vec{k}_z$ is purely imaginary, corresponding to an electric field decaying exponentially with distance from the interface. The probe depth, δ_z, and L_x are defined as the distances (along z or x, respectively) at which the intensity of the electric field has dropped to a value of $1/e$ and are hence given by ($''$ denotes the imaginary part)

$$L_x = \frac{1}{2\vec{k}_x''}, \tag{4}$$

$$\delta_z = \frac{1}{2\vec{k}_z''}. \tag{5}$$

The propagation length of the surface plasmon governs the lateral resolution of iSPR. Consequently all features smaller than L_x will be blurred along the direction of propagation while the perpendicular resolution is limited by diffraction. Equations (4) and (5) are strictly valid only for the case of two adjacent semi-infinite media. If we take into account that the metal film is generally thin (typically 50 nm), the surface plasmons are subject to radiative damping. For resonantly excited surface plasmons, the radiative damping is approximately equal to the heat dissipation and an appropriate estimate of the propagation length is to take $L_x = 1/4\vec{k}_x''$. For a thin gold film, at a wavelength of 690 nm, this gives a propagation length of about 10 μm. The probe depth, δ_z, into the dielectric accounts for the surface sensitivity of surface plasmon resonance, which at a wavelength of 690 nm is about 120 nm.[12] Thus, the attachment organs of the exploring cyprids are only visible in the surface plasmon resonance image when they are within a few hundred nanometers from the surface.

In the custom built iSPR instrument used in this work, schematically outlined in Fig. 1, glass slides coated with a thin layer of gold (provided by GE Healthcare, Biacore division) were optically coupled to a glass prism (BK7 glass, Melles Griot) using a refractive index matching oil (Cargille-Sacher Laboratories Inc.). Light from a white light source was passed through a monochromator, collimated and TM-polarized before reaching the prism at a specific angle of incidence, θ (72.5°). The intensity of the reflected light was measured using a charge coupled device camera (Retiga EXi, Qimaging Corp., 12 bit 1 Mpixel without IR filter). At the set angle of incidence, each pixel in the image depicted an area of 2.2×5 μm^2. Also shown in Fig. 1 is a cuvette with a 0.5 ml open compartment which was used to confine cyprids during the experiments. 15 cyprids were added to the cuvette in 33 PSU artificial seawater and allowed to explore the substrate for 7 min. During this time SPR images were captured at a rate of 1 Hz. During cyprid exploration, the wavelength was fixed at 690 nm and the acquired images were normalized with respect to the reflection of TE-polarized light. An image acquired before addition of cyprids was used as base line and subtracted from all subsequently captured images. To yield quantitative data, the wavelength was scanned be-

tween 650 and 800 nm and the SPR wavelength was determined for each image pixel before and after cyprid exploration.

Previous fouling resistance studies of model systems based on oligo(ethylene glycol) (OEG) containing self-assembled monolayers (SAMs) or hydrogels have shown cyprid and algal settlement to be consistently low on such surfaces.[13,14] However, while the inherent fouling resistance of OEG SAMs is well known, its mode of action has not been elucidated. First and foremost, it remains unknown whether the fouling resistance of OEG is the product of voluntary rejection by the cyprids, based on unfavorable surface characteristics, or whether the attachment method is precluded by OEG in many cases. The ability to monitor the exploratory behavior and substrate-organism interactions on these surfaces could therefore offer a means to interpret settlement observations in context.

Figure 2 presents selected SPR images, representative for a triplicate set of trials, from image sequences acquired at fixed wavelength (690 nm) during cyprid exploration on a gold substrate covered with a SAM of $HS(CH_2)_{11}CONH(C_2H_4O)_{11}CH_3$ (mOEG) and a bare gold substrate, respectively. On both surfaces, the cyprids were observed to probe the substrate with their antennules, however, footprints are accumulated only on the bare gold. The arrows in each image indicate current surface contact points between the attachment disks of exploring cyprids and the substrate. From the image series in Fig. 2 it can be seen that although the cyprids clearly probe the mOEG substrate, very little proteinaceous residue remains at the contact points once the attachment organ is withdrawn. On the bare gold substrate, however, the deposited footprint remains on the surface once the cyprid has moved on, leading to significant accumulation of bright spots in the iSPR image over time. In both cases the size of the antennule-substrate contact point is about 50 μm and the stride is a few hundred micrometers.

While iSPR images of the type shown in Fig. 2 can be used to yield comprehensive data on the exploratory behavior of fouling organisms *in situ*, interpretation of the SPR wavelength shifts relating to the observed adhesive deposits may also allow quantification of the material remaining on the explored surface. Such quantitative data are presented for the evaluated gold and mOEG surfaces in Fig. 3. As exemplified in the reflectivity curves (acquired from the encircled regions in Fig. 3), the brightest spot on the gold surface corresponds to a SPR wavelength shift of some 28 nm. In contrast, for a corresponding region on the mOEG substrate, where an exploring cyprid antennule had been observed, there was no discernable shift in SPR wavelength. The interpretation of these data would be that footprints of proteinaceous material were deposited postexploration on gold, whereas on mOEG, despite seemingly similar exploratory behavior, they were not. This would suggest that the resistance of mOEG to footprint deposition is more likely to be due to the antifouling properties of the surface than any voluntary alteration in the process of exploration by the cyprids.

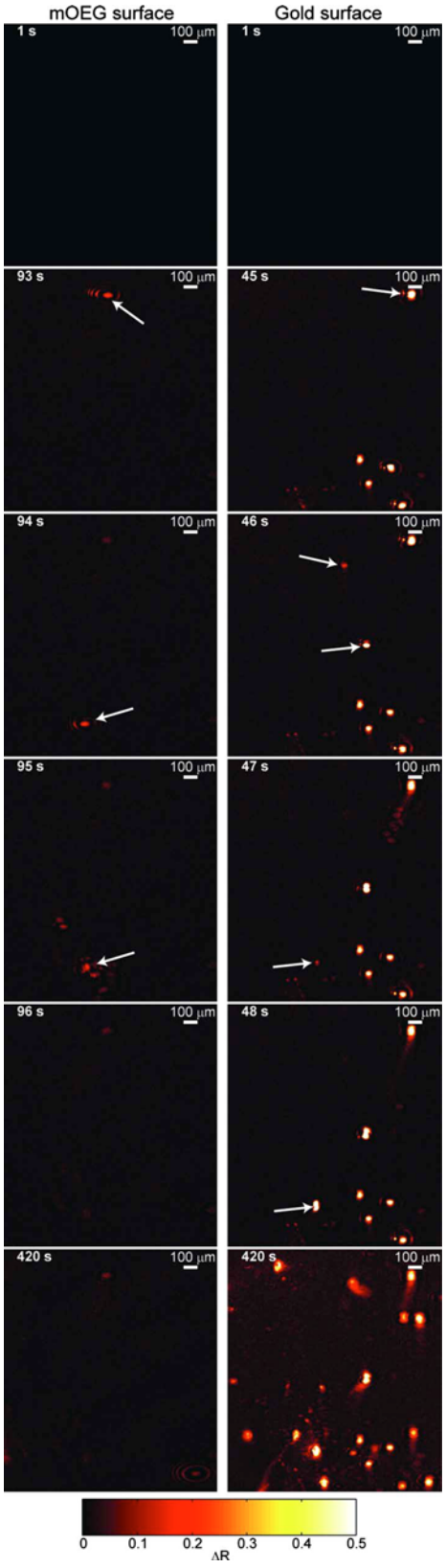

FIG. 2. (Color online) Snapshots of the mOEG and gold surfaces selected from a time series of SPR images acquired during cyprid exploration. Cyprid antennules close to or touching the surface are visible, as bright spots in the SPR images. The white arrows highlight the surface-antennule contact points in each frame. Deposited proteinaceous material is visible as accumulating spots, particularly on the gold surface. The full image series is available as supplementary material (video).

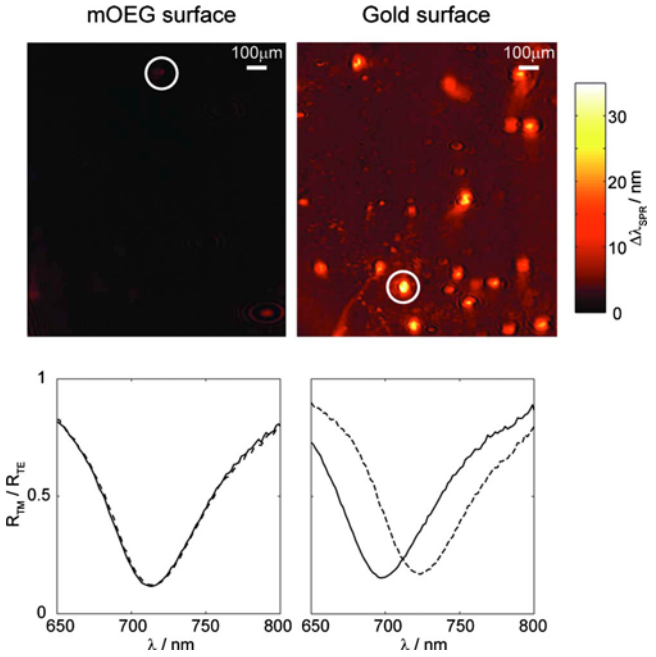

FIG. 3. (Color online) Imaging SPR wavelength maps showing the shift in SPR wavelength ($\Delta\lambda_{SPR}$) for a mOEG and a gold surface after 7 min of cyprid exploration. The reflectivity curves were acquired from within the encircled regions before (solid) and after (dashed) exposure to cyprids. Cyprid antennules were observed to touch the surface within the selected regions on both substrates during exploration.

It is also possible to calculate a corresponding surface coverage of proteinaceous material on explored surfaces using the conventional estimation of a refractive index of $n=1.45$ for an adsorbed protein film. The instrumental sensitivity ($\delta\lambda/\delta d$) to changes in thickness of an adsorbed organic film can then be rigorously calculated using the Fresnel theory. For our iSPR instrument, under the current experimental conditions, the SPR wavelength shifts about 2 nm for every nanometer of deposited material.[15] A shift in SPR wavelength of some 28 nm, as on the gold surface, thus corresponds to an adsorbed protein film of 14 nm. The thickness of footprints from *S. balanoides* has been previously estimated, by atomic force microscopy, to fall between 5 and 19 nm, depending on the surface.[4]

In future applications of this technique, we will evaluate the behavior and adhesive interactions of cyprids on a diverse range of substrates with controlled chemistry before continuing with other test species. In time, it is expected that this approach will yield important information regarding the adhesion of such organisms to specific substrates and, thus, inform the development of surfaces to which settlement is impossible. This technique could also be combined with visualization methods capable of three-dimensional tracking above the surface, such as in-line holography,[16] to enable full insights in the moving pattern and surface interactions of the exploring organisms.

Imaging SPR time lapse videos of exploring cyprids are available for download from the journal homepage.[17]

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