Surface Plasmon Resonance/Surface Plasmon Enhanced Fluorescence

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Multi Component Macromolecular Adsorption and Reaction Study by Surface Plasmon Resonance/Surface Plasmon Enhanced Fluorescence

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Introduction

The adsorption of macromolecules to solid surfaces is of importance in a wide variety of technologies, both biological and otherwise. Polymer adsorption is used in such applications as pre-coating surfaces in biosensors and surface modification for adhesion and lubrication[1]. The adsorption of biological polymers, or proteins, plays a role in separation processes and is a limiting factor in the design of virtually any biocompatible material. For example, when blood comes into contact with a biomedical device, various plasma proteins, including albumin, immunoglobulins, and fibrinogen adsorb to the surface and thereby establish the extent of surface biocompatibility[2,3]. A precise understanding of the physics of adsorption is necessary to design a surface that is most biocompatible. A particularly interesting aspect of proteins is the potential for retention of their intrinsic function when adsorbed to a surface. Thus, surface bound antibodies may retain their specificity for antigens, ligands may still bind their proper proteins, and enzymes may catalyze biological reactions. This surface activity is exploited in such applications as diagnostic devices, contact lens cleaners, and detergent enzyme additives.

For these reasons, a large volume of work is available on the adsorption of proteins to solid surfaces. Much is now known about the surface properties that govern both the equilibrium and kinetics of adsorption of single component. However, in multi component systems, several components are present at the surface at once and thus there may be several events occurring simultaneously. De-convoluting such events with a single experimental technique can often be a complicated process and is nearly impossible to do in real time.

One solution to this problem would be to use two or more techniques simultaneously to distinguish multiple components at the surface.[4] We use a tandem technique for the measurement of simultaneous surface events, in which both techniques, surface plasmon resonance (SPR) and surface plasmon enhenced fluorescence (SPEF), rely on the resonant excitation of surface plasmons.[5,6]

The relative ease of construction and linear correlation between SPR and SPEF signals make the technique ideal for coadsorption studies or multiple ligand binding experiments. Here, we demonstrate the utility of the technique with a biotin/avidin/BSA sandwich experiment. We then apply SPR/SPEF for the simultaneous monitoring of enzyme adsorption and substrate cleavage of a protease interacting with a substrate surface.

Materials and Methods

Apparatus construction

A schematic of the experimental setup is shown in Figure 1. A 1mW He-Ne laser is directed by a series of mirrors through a neutral density filter (used to control incident light intensity) and into the optical train. The beam passes through a Glan-Thomson polarizer to ensure p-polarized light for the SPR experiment. A spatial filter removes stray light from the beam, yielding a more homogeneous profile, followed by a beam collimator/expander which expands it to the appropriate diameter (~1 in.). The widened beam is focused onto the sample cell by a vertically mounted planar cylindrical lens. An entire SPR spectrum can thus be captured at once on the CCD. In our experiment, we capture a horizontal spectrum over an angular width of ~ 8°. The reflected beam is "vertically averaged" by a horizontally mounted cylindrical lens and directed onto the CCD element of a 1D CCD, which records the reflectivity data. Fluorescence is measured from behind the sample cell using a PMT.

Sample Cell

　　　　A thin 50 nm gold film with a 2nm chromium undercoat is evaporated onto a polished SF10 glass (n=1.723) slide.　The surface is given the desired functionality by means of a thiol anchor carrying the desired reactive group.　Another glass slide is sandwiched together with the first, with the gap between them defined by a silicone gasket (thickness 0.5 mm).　A solution containing the desired analyte is then flowed through the cell by means of a peristaltic pump.

　　　　The entire assembly is mounted onto an SF10 glass (n=1.723) hemi-cylindrical prism.　Index matching is accomplished via a matching liquid (n=1.730).　This provides a close but not perfect match to the SF10 glass and as a result, we observe an interference pattern on our image on the CCD.　We eliminate the effects of this pattern by vertically averaging the image using a second focusing lens just before the camera.
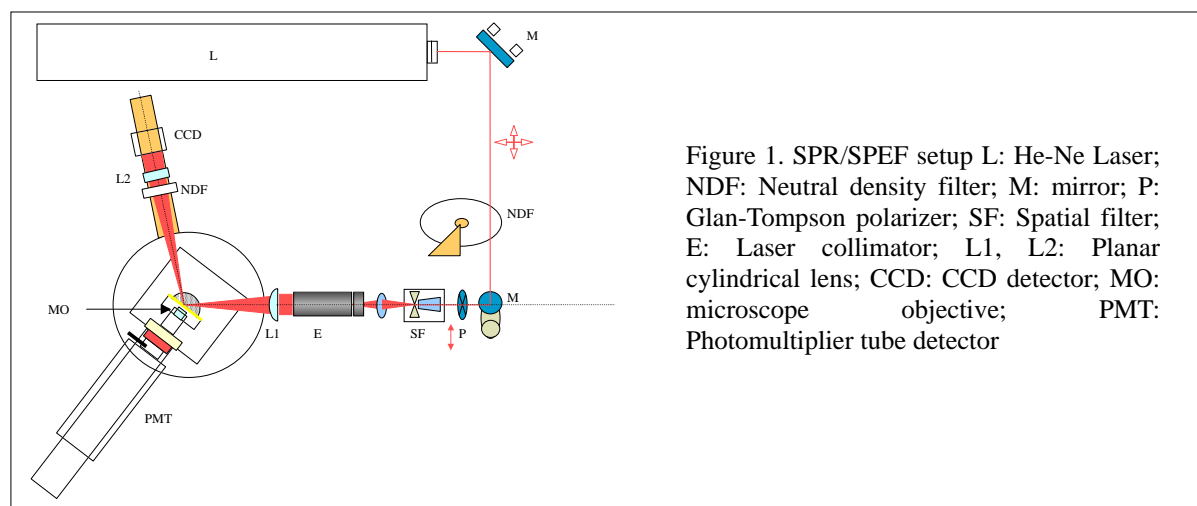

Figure 1. SPR/SPEF setup L: He-Ne Laser; NDF: Neutral density filter; M: mirror; P: Glan-Tompson polarizer; SF: Spatial filter; E: Laser collimator; L1, L2: Planar cylindrical lens; CCD: CCD detector; MO: microscope objective; PMT: Photomultiplier tube detector

Substrate Surfaces

　　　　Self assembled monolayers of propionic acid were prepared by dipping gold-coated slides into a 3-mercaptopropionic acid solution. The carboxyl end groups of the immobilized hydrocarbon chains were then activated for peptide bond formation using EDC (1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide hydrochloride) / NHS (N-hydroxysuccinimide) chemistry. The EDC/NHS step produces a water stable ester able to react with primary amines.　Biotinylated surfaces were produced by reaction with biocytin hydrazide in reaction buffer.　Monolayers of covalently bound fluorescent tagged bovine serum albumin (BSA) were formed by reaction with Texas Red/BSA conjugates in reaction buffer.

Protein Solutions

　　　　Biotin was conjugated to BSA in a manner identical to that used to biotinylate the surface. Carboxy-side chains of BSA were activated by reaction with EDC and NHS then covalently linked to the amine group in biocytin hydrazide.　Conjugates were separated from unreacted biotin using gel filtration chromatography.　*B. lentus* subtilisin enzyme was obtained from Genencor International.

Results and Discussion

Avidin-Biotin Sandwich Experiment

　　　　To demonstrate the utility of the SPR/SPEF technique in distinguishing between multiple components at a surface, we conducted a set of biotin/avidin/BSA sandwich experiments.　In each case, we start by binding a monolayer of biotin to the functionalized layer of hydrocarbons.　In the first experiment, we allow a monolayer of fluorescently labeled avidin to bind the biotin.　The SPR and SPEF signals are proportional to one another. (data not shown)

　　　　In the next experiment, we successively add labeled and unlabeled avidin to the monolayer of biotin. The SPR signal rose throughout both additions due to a continued increase in protein layer thickness; however the SPEF signal only rose following addition of the labeled protein. (see Figure 2)　Thus, the two signals can be used to separate labeled and unlabeled species.　The same idea can be used to distinguish the presence of two

separate proteins on the surface. The corresponding control experiment is shown in Figure 2b. In this case, labeled avidin is added in both steps demonstrating no distinction between SPR and SPEF signals.
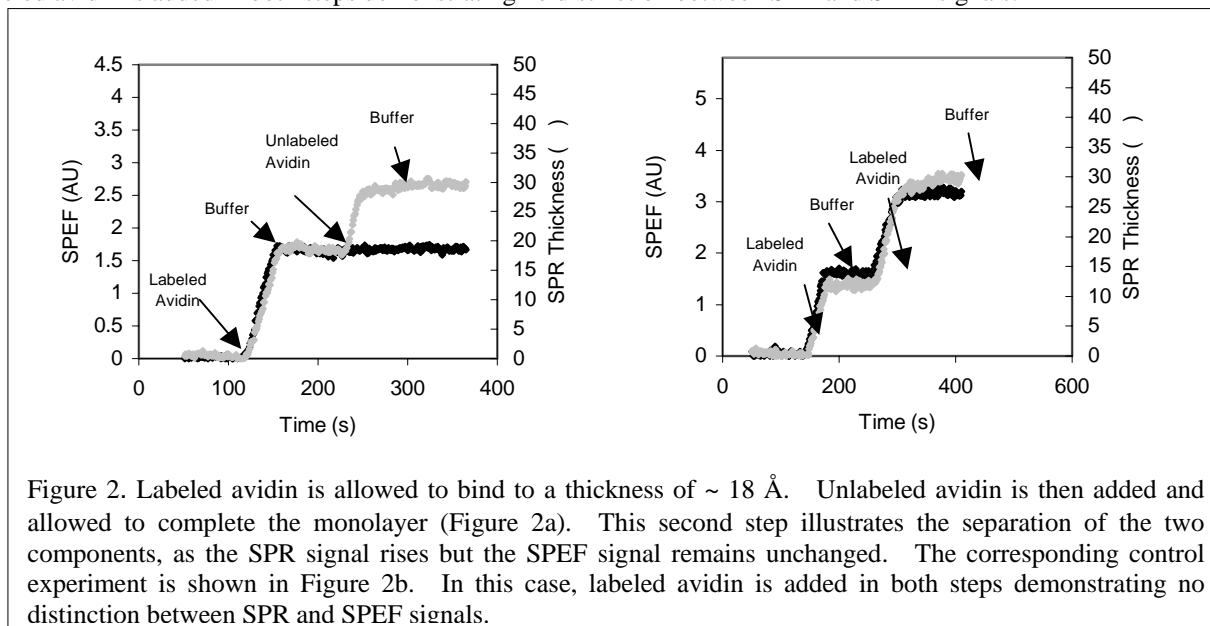

Figure 2. Labeled avidin is allowed to bind to a thickness of ~ 18 Å. Unlabeled avidin is then added and allowed to complete the monolayer (Figure 2a). This second step illustrates the separation of the two components, as the SPR signal rises but the SPEF signal remains unchanged. The corresponding control experiment is shown in Figure 2b. In this case, labeled avidin is added in both steps demonstrating no distinction between SPR and SPEF signals.

Application to Measurement of Adsorption and Reactivity

Our primary goal in developing this technique is to simultaneously measure the adsorption and reaction kinetics of an enzyme interacting with a substrate surface. Our model substrate for this experiment is fluorescently labeled BSA. The enzyme is the serine protease subtilisin. Subtilisin adsorbs to and cleaves BSA from the surface.

Figure 3 shows a result from this experiment. At the beginning of the experiment, buffer is passed over the surface to establish an initial gold/buffer surface plasmon. Once enzyme solution is added, it begins to hydrolyze the BSA layer. The drop in the SPR signal indicates a thinning of the total protein layer. This results from a combination of enzyme adsorption (the layer thickens) and BSA hydrolysis (the layer is depleted).
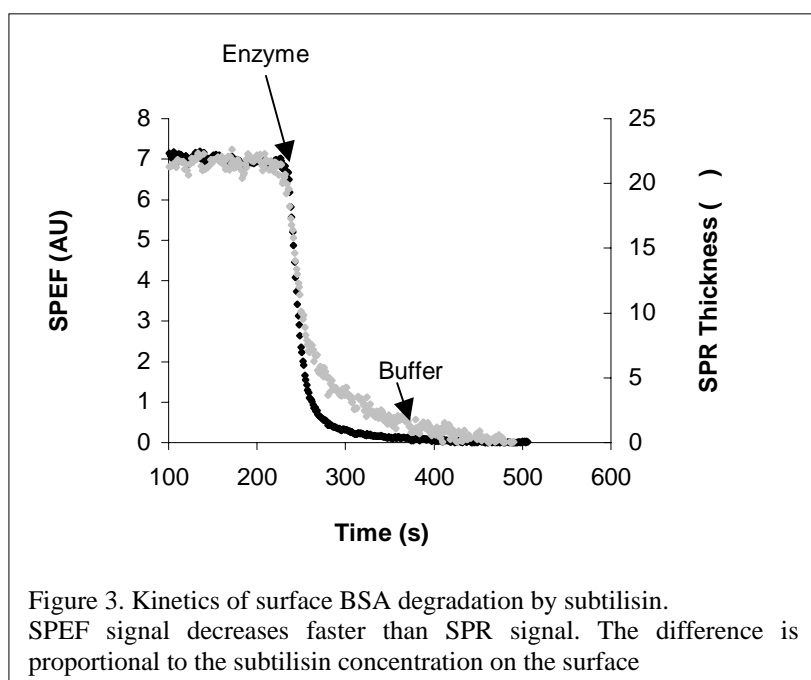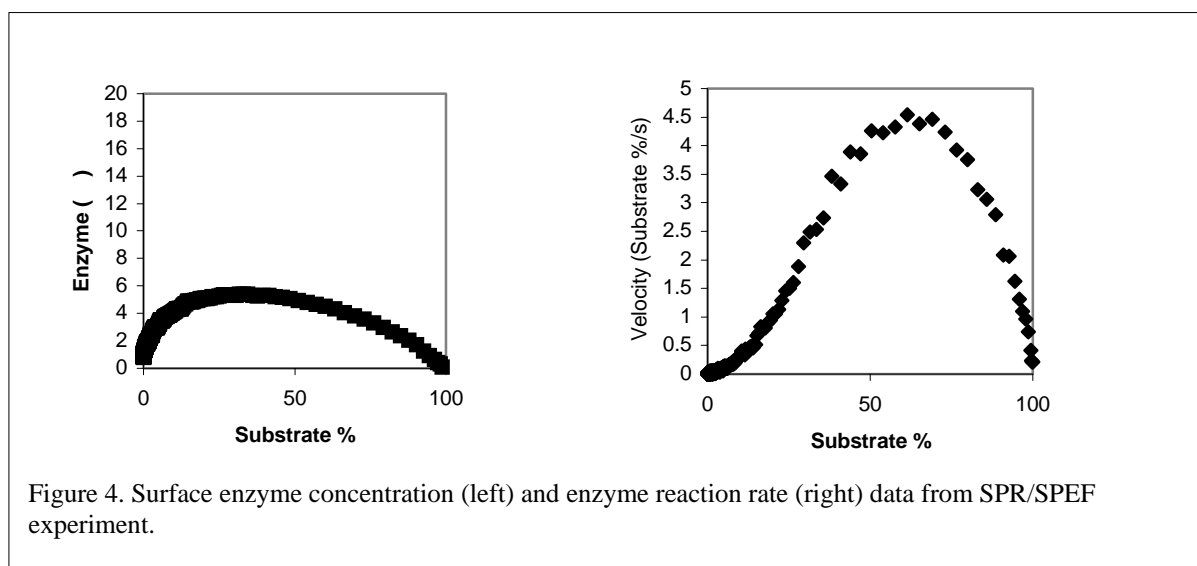

Figure 3. Kinetics of surface BSA degradation by subtilisin. SPEF signal decreases faster than SPR signal. The difference is proportional to the subtilisin concentration on the surface

The fluorescence signal isolates the loss of BSA, or the kinetics of the reaction. Once again, taking advantage of the linearity between the SPR and SPEF signals, the displacement between the two signals can be taken to isolate the kinetics of adsorption. As shown in Figure 4, we are thus able to observe the dynamic adsorption and reactivity behavior of an enzyme interacting with a substrate surface. A complete analysis of our adsorption /reaction results including kinetic modeling is the subject of a subsequent report.

Figure 4. Surface enzyme concentration (left) and enzyme reaction rate (right) data from SPR/SPEF experiment.

Conclusion

The surface properties of macromolecules are often difficult to discern due to the simultaneous presence of multiple species at the surface.   Using the tandem technique of SPR/SPEF the amounts of each individual species on the surface can be determined.   We demonstrated the utility of the technique by distinguishing two separate proteins using a biotin/avidin/BSA "sandwich" assay.   SPR/SPEF can also be used to probe more complicated dynamic behavior, as illustrated by the measurement of concurrent adsorption and reaction of an enzyme interacting with a substrate surface.   In such cases, simultaneous surface events are often co-dependent, and using SPR/SPEF, one can observe the interplay of competing phenomena.

Acknowledgement

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