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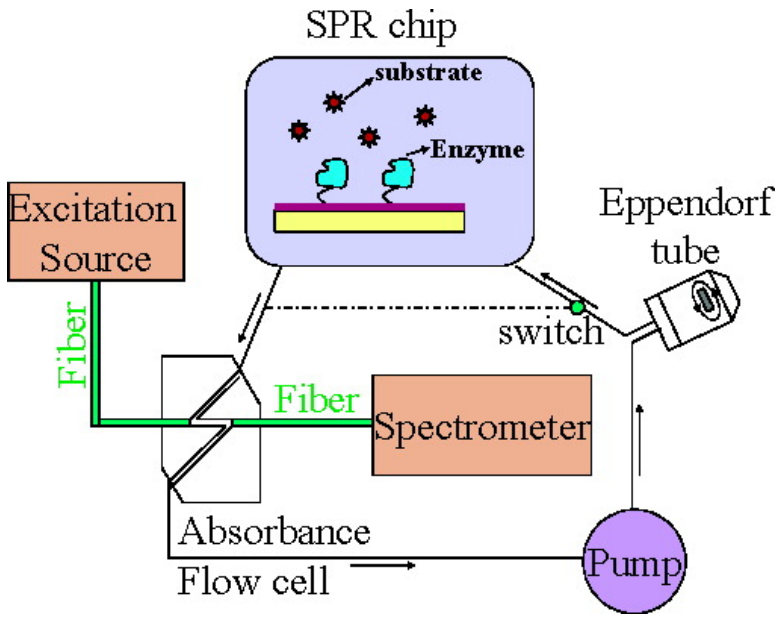

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Combined Affinity and Catalytic Biosensor: In Situ Enzymatic Activity Monitoring of Surface-Bound Enzymes

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A biosensor is a device consisting of a biofunctional surface coating and a transducer, which relates the concentration of an analyte or mixture of analytes to a measurable response. Generally, there are two categories of biosensors, affinity biosensors and catalytic biosensors.[1] The transducer can be electronic, optical, electrical, etc. In this study, the combination of the two different types of biosensors both based on optical transduction principles is presented. Optical biosensors offer advantages in terms of miniaturization, low cost, disposability, and lack of electrical interference. A home-built Surface Plasmon Resonance Spectrometer (SPR) and a commercial miniature Fiber Optic Absorbance Spectrometer (FOAS) served as a combined affinity and catalytic biosensor. A model enzyme was covalently attached to the SPR chip to catalyze the turnover of a substrate, which was detected in situ with the FOAS system.

SPR is a widely accepted analytical tool for the characterization of interfaces and thin films and is used here to monitor the surface coverage of biotin-β-lactamase on the surface of a chip. β-Lactamase was chosen as a model enzyme because of its known three-dimensional structure and high catalytic efficiency, which allows for the quantification of enzyme activity at the femtomole range.[2] The β-lactamase was genetically engineered to contain a single cystein at a specific site on the surface of the enzyme that could be biotinylated with the aim to immobilize the enzyme molecules in defined orientations in relation to its active site. Details of this strategy are given in ref 3. The model system (Figure 1A) used in the study was based on supramolecular interfacial architectures with NeutrAvidin as a universal binding matrix.[3] A binary mixture of biotin-terminated thiols and hydroxyl-terminated diluent thiols was assembled onto chromium/gold films from ethanolic solution to form a biotinylated Self-assembled Monolayer (SAM).[4] Once the binding of a NeutrAvidin monolayer with molecularly controlled orientation is established, the remaining free binding sites exposed to the aqueous phase were used to immobilize in a subsequent step the biotinylated enzyme. The biotin-β-lactamase utilized in the study had a single biotin group linked through a 2.9 nm long spacer that contained a disulfide bridge.[3] SPR was used to monitor in situ and in real time the kinetics of the interfacial processes. Different surface coverages of the enzyme were obtained by adding biotinylated β-lactamase at different solution concentrations to the NeutrAvidin monolayer coated surfaces, followed by rinsing with pure buffer (Figure 1B). Table 1 lists the quantitative surface immobilization data of β-lactamase. On the basis of Fresnel analysis, the optical thickness of biotin-β-lactamase (assuming a refractive index of $n = 1.41$) can be calculated.[5] The concentration of the surface-immobilized enzyme was deduced from the adsorbed mass, converted according to de Feijter et al.'s formula[6] from the optical thickness using a refractive index increment (dn/dc) of 0.18 cm^3/g for β-lactamase.[3]

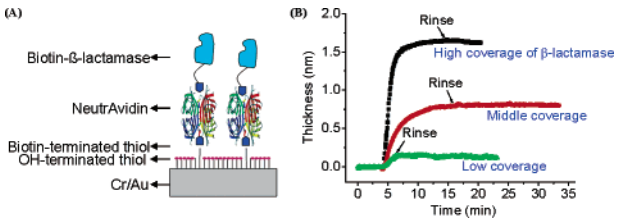

Figure 1. (A) Cartoon of the interfacial architecture of the model system used for the SPR/FOAS investigation. (B) SPR kinetics of the immobilization of biotin-β-lactamase on the NeutrAvidin-functionalized chip at different β-lactamase solution concentrations, resulting in different enzyme surface coverages.

Table 1. SPR Results and Kinetic Parameters Obtained from Miniature Fiber Optic Absorbance Spectroscopy (FOAS) for Surface-Immobilized and Free β-Lactamase (numbers are mean values $\pm$ standard deviation)

	Enzyme Immobilized on SAM/Au substrate			"free" enzyme
biotin-β-lactamase	high	medium	low	in solution
thickness (nm)[a]	1.6	0.8	0.13	
C (n mol L^{-1})[b]	6.98	3.45	0.56	0.3
K_M (10^{-6} mol L^{-1})[c]	1070 $\pm$ 340	280 $\pm$ 80	120 $\pm$ 30	200 $\pm$ 20
k_{cat} (s^{-1})[d]	152 $\pm$ 42	90 $\pm$ 17	149 $\pm$ 20	814 $\pm$ 71
k_{cat}/K_M (10^6 mol^{-1} L s^{-1})[e]	0.14 $\pm$ 0.03	0.32 $\pm$ 0.06	1.24 $\pm$ 0.17	4.07 $\pm$ 0.36

[a] Thickness, obtained from Fresnel simulation. [b] C, the concentration of surface-immobilized β-lactamase involved in the catalysis of substrate inside the SPR flow cell. [c] K_M, Michaelis constant, the substrate concentration at which the reaction velocity is half-maximal. [d] k_{cat}, turnover number, the number of reaction events per enzyme molecule and second. [e] k_{cat}/K_M, catalytic efficiency of the enzyme.

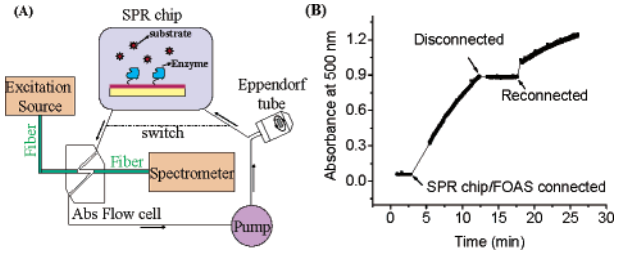

Figure 2. (A) Schematic of the FOAS setup for the enzymatic activity assay. (B) Kinetics of the turnover of nitrocefin detected with FOAS for the investigation of surface-bound β-lactamase monitored at $\lambda = 500$ nm.

The basic element of the FOAS sensor (Figure 2A) is a special flow cell, which couples the substrate solution flowing through the cell with the surface-immobilized β-lactamase of the SPR sensor to the FOAS system. Two optical fibers were used for excitation and detection, respectively. A peristaltic pump was used to circulate the substrate solution through the loop. The activity of β-lactamase

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was measured quantitatively through monitoring the reaction with the chromogenic substrate nitrocefin,[7] which undergoes a distinctive color change from yellow (λ_{max} = 390 nm at pH 7.4) to red (λ_{max} = 486 nm at pH 7.4) as the amide bond in the β-lactam ring is hydrolyzed by β-lactamase. The maximum absorbance of the nitrocefin product shifted from λ = 486 to λ = 500 nm upon the addition of bovine serum albumin (BSA), which proved to be necessary for stabilizing low concentrations of β-lactamase in solution.[3] After recording the dark signal and the reference signal with pure buffer, the nitrocefin solution was first injected into the FOAS system for several minutes to establish a baseline. Subsequently, the solution was allowed to run through the SPR flow cell. The conversion of the nitrocefin by the catalytic action of biotin-β-lactamase immobilized on the SPR sensor surface was monitored through changes of absorbance at λ = 500 nm as a function of time at room temperature. A relative standard deviation of 2% for the initial rate of absorbance change measured repeatedly demonstrates the excellent reproducibility of the enzymatic activity assay used in this study (details not shown).

Enzyme activity and stability are usually key factors in determining the lifetime of an enzyme-based biosensor chip. Figure 2B demonstrates the turnover of nitrocefin by the chip-bound enzyme as a function of time. The immobilized biotin-β-lactamase showed significant enzymatic activity when the substrate solution passed through the SPR flow cell. If the connection between SPR flow cell and the FOAS flow cell was interrupted, the turnover of nitrocefin ceased immediately. If the connection between the two systems was re-established, the enzymatic turnover recovered on a similar level as before. We conclude from these observations that the measured enzymatic turnover is entirely due to the specific action of the surface-bound enzyme molecules, implying that desorption of enzyme molecules into the solution does not take place to a measurable degree.

The activity of β-lactamase was also tested in solution utilizing the FOAS, the result serving later as a control for the evaluation of the kinetic activity of the surface-immobilized enzymes. A set of kinetic runs with surface-bound β-lactamase at different enzyme surface coverages was performed with the substrate nitrocefin and analyzed in the framework of the Michaelis−Menten formalism.[3] The values of the kinetic parameters K_M and k_{cat} determined by measuring the initial reaction velocities at various substrate concentrations are summarized in Table 1. The relatively high standard deviations are due to the limited nitrocefin concentration range accessible in the Michaelis−Menten experiment as a result of limited nitrocefin solubility. To determine the catalytic efficiency, k_{cat}/K_M, of an immobilized enzyme, the exact amount of enzyme on the surface has to be known. For this purpose, SPR served as an independent technique to determine the interfacial enzyme concentration. The data in Table 1 demonstrate that all k_{cat} and k_{cat}/K_M values, corresponding to turnover number and enzyme efficiency, respectively, of surface-immobilized β-lactamase (with high, medium, and low surface coverage) were significantly lower than those of the enzyme in solution using nitrocefin as the substrate. Several factors may contribute, in principle, to the observed reduction in enzymatic activity, in particular, partial denaturation of enzyme upon immobilization and substrate diffusion to the interface. A gradual reduction in the K_M values was observed with decreasing enzyme surface coverage, resulting from the decreased protein surface coverage accelerating the recognition process of the enzyme and substrate. K_M values of the immobilized β-lactamase with medium and low coverage were similar to that of the enzyme in solution, which shows that diluted surface-immobilized enzymes and free enzymes in solution have similar enzyme−substrate affinities. In conjunction with the fact that the k_{cat} values, which indicate the ability of product formation and release from the active site, of surface-immobilized enzymes with different binding densities are quite similar, we confirmed that the observed reduced enzymatic activity for surface-attached enzymes was mainly due to the steric crowding effect of the enzymes and the resulting restricted substrate accessibility of the enzyme active site.

There are a number of advantages for the combination of SPR and FOAS techniques. The parallel measurement offers the opportunity of on-line activity detection for surface-attached enzymes. The immobilized enzyme does not have to be in contact with the catalytic biosensor. The SPR chip can easily be cleaned and used for recycling. Additionally, with regard to the application of FOAS, the integrated SPR technique allows for the quantitative control of the surface density of the enzyme, which is highly relevant for the enzymatic activity. Finally, the miniaturized portable FOAS devices can easily be combined as an add-on device with many other in situ interfacial detection techniques, such as optical waveguide lightmode spectroscopy (OWLS), quartz crystal microbalance (QCM) measurements, or impedance spectroscopy (IS).

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Supporting Information Available: Materials, surface modification for SPR measurement, assembly of FOAS system, enzymatic activity assay, and Michaelis−Menten analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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