Surface plasmon optical detection of β-lactamase binding to different interfacial matrices combined with fiber optic absorbance spectroscopy for enzymatic activity assays

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In this study, we describe the attachment of biotin-functionalized β-lactamase to different types of interfacial architectures. Generic biotin-NeutrAvidin binding matrices were assembled using biotin-terminated alkanethiol and poly (L-lysine)-*g*-poly (ethylene glycol) polymer. Quantitative comparisons were made between different matrices and binding strategies. In addition, the feasibility of regeneration was tested. Our results show that in general all matrices were well suited for the binding of the protein, although quantitative differences were observed and will be discussed. Furthermore, the results obtained by surface plasmon resonance spectrometer and optical waveguide measurements show excellent correlation. For all five matrices investigated, real time enzymatic activity assays of β-lactamase were performed by a detection scheme that combines an affinity and a catalytic sensor. The results show that the surface-immobilized enzymes are stable and sufficiently active for highly sensitive catalytic activity measurements. The effect of surface immobilization on the catalytic activity of the enzyme is discussed.
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I. INTRODUCTION

Biosensors, consisting of a molecular recognition element and a transducer, are contributing to diverse scientific fields and are used to examine interactions between partners ranging in size from small molecules to whole cells.[1] A recently established biosensor device,[2] combining a surface plasmon resonance spectrometer (SPR) and a commercial miniature fiber optic absorbance spectrometer (FOAS), was employed in a detection scheme designed for the quantitative *in situ* evaluation of the degree of binding and the catalytic activity of surface-immobilized enzymes. SPR relies on the measurement of binding-induced refractive index changes and thus is a prominent example of label-free detection principles. Over the past ten years, SPR biosensor technology has made great progress, resulting in the development of a large number of SPR sensor platforms, biomolecular recognition elements, and measurement formats.[3] Miniature FOAS, on the other hand, has been described in detail in a previous report.[2] Briefly, light from a halogen lamp reaches a special FOAS 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. The spectrum of the transmitted light is detected by a miniature fiber optic spectrometer. The parallel measurements offer the opportunity for on-line activity detection of 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 reused for repeated assays. Furthermore, with regard to the application of FOAS, the combination allows for the quantitative control of the surface density of the enzyme by the SPR technique, which is highly relevant for enzymatic activity assays.

To be quantitative and efficient, sensors need to be rapid, specific, reproducible, and highly sensitive. Furthermore, they need to have an optimized density of highly selective and specific biofunctional groups for the recognition (and binding) of the analyte molecule of interest. Improved strategies for the immobilization of biologically active recognition elements have led in the past years to a substantial increase in sensitivity and selectivity of bioaffinity sensor devices. Controlling the immobilization of biomolecules on surfaces, while preventing nonspecific adsorption of undesired species, has become an important goal for monitoring specific biointeractions and binding of biomolecules or cells. Therefore, in the past decades, many immobilization strategies have been established. Attempts to control the density, orientation and presentation of biomolecules at the solid-liquid interface for better sensitivity and reproducibility have been undertaken through various strategies for covalent and site-specific immobilization.

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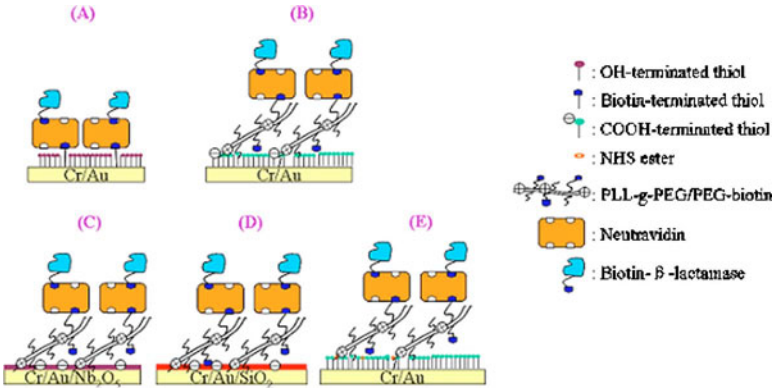

FIG. 1. Schematic of the five matrices used for protein binding.

In this paper, five different types of interfaces (cf. Fig. 1) were investigated based on a generic biotin-NeutrAvidin binding matrix for the quantitative control of the degree of enzyme binding, as well as for measuring the enzymatic activity of biotin-β-lactamase. Biotin and avidin analoges exhibit one of the strongest noncovalent affinity interactions in nature ($K_A = 10^{13-15}$ M^{-1} in solution), and as such have been used for a wide range of applications including immunoassays, cytochemistry, protein purification, and diagnostics.[4]

The first example of the five matrices is a self-assembled monolayer (SAM), biotin-terminated alkanethiol, onto which NeutrAvidin was immobilized. Once the binding of a molecularly ordered NeutrAvidin monolayer was established, the remaining free binding sites exposed to the aqueous phase were used to build subsequent layers. This kind of a well-organized and relative stiff matrix was used as a control for the other four kinds of highly flexible matrices used in the study. Poly (L-lysine)-g-poly (ethylene glycol) (PLL-*g*-PEG) is a polycationic copolymer that adsorbs spontaneously from aqueous solutions onto negatively charged surfaces via electrostatic or covalent interactions. It forms monolayers with densely packed, brush-like and highly flexible PEG chains, which extend toward the aqueous solution. In addition, the use of PEG tethers for biomolecular immobilization has been reported to minimize loss of protein activity.[5] PLL-*g*-PEG graft copolymers carrying terminal biotin groups (PLL-*g*-PEG/PEG-biotin) were synthesized and used for the buildup of capture matrices for proteins. These biotinylated, PEG-brush surfaces have been shown to resist nonspecific adsorption from serum while still allowing for the specific surface binding of avidin, NeutrAvidin, or streptavidin and the subsequent immobilization of biotinylated biomolecules such as biotinylated antibodies.[4]

As an example, carboxyl-terminated alkanethiols were assembled on a gold surface in order to form a negatively charged surface that served as a substrate for the subsequent polymer adsorption via either electrostatic or covalent binding. For comparison, gold substrates were coated with thin metal-oxide layers consisting of niobium oxide (Nb_2O_5) or silicon oxide (SiO_2), onto which polycationic polymeric adlayers were directly adsorbed by electrostatic interactions. Owing to the fact that high-refractive-index, transparent oxides of transition metals such as niobium or tantalum, are suitable as waveguiding layers for optical evanescent-wave-based sensing devices, the deposition of such metal oxide layers on top of a noble metal offers the potential of using surface plasmon (SPR) spectroscopy, optical waveguide lightmode spectroscopy (OWLS), and plasmon-waveguide resonance spectroscopy, in particular. This way, results obtained by the different methods can be compared directly.

Enzymes are one of the most commonly used biological components of biosensors. They catalyze a large number of reactions and thus offer the possibility of detecting a broad range of analytes (e.g., substrates, products, inhibitors, or modulators of the catalytic activity).[6] Because enzymes are natural proteins that transform a specific substrate molecule into a product without being consumed in the reaction, they can easily be used for the continuous and repeated sensing of a specific compound. We chose β-lactamase[7,8] as a model enzyme because its three-dimensional structure is known and it exhibits an extraordinarily high catalytic efficiency, which allows for the quantification of enzyme activity in the femtomole range.[9] β-lactamases are the most common reason of bacterial resistance to β-lactam antimicrobial agents. The impact of β-lactamase has already been enormous, and its potential to challenge antimicrobial chemotherapy remains promising.[10] A specific amino acid such as a single cysteine residue has been introduced at a surface-exposed position of β-lactamase by site-directed mutagenesis. The unique SH group on the protein surface can then be selectively modified with ligands via thiol-specific chemistry. Biotin-β-lactamase was thus obtained by derivatizing the surface-exposed cysteine with a dithiothreitol (DTT)-cleavable biotinylation reagent for the specific attachment to the interface. Exposure to DTT was used to quantitatively release the biotin-β-lactamase from the chip.[11]

In this study, surface plasmon resonance (SPR) spectroscopy and optical waveguide lightmode spectroscopy (OWLS) were used to monitor quantitatively and *in situ* the spontaneous adsorption of polymers, as well as proteins, onto the sensor surface (cf. Fig. 1), and the possibilities for regeneration. For the detection of the activity of the enzyme immobilized on the five different matrices as well as in solution, a miniature fiber optic absorbance spectroscopy (FOAS) system was assembled for the detection of a chromophoric reaction catalyzed by β-lactamase. We performed

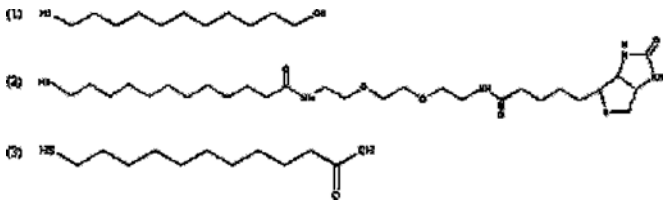

FIG. 2. Structure formula of (1) hydroxyl-terminated thiol (11-Mercapto-1-undecanol), (2) biotin-terminated thiol (HS-c12-dadoo-Biotin), and (3) carboxyl-terminated thiol (11-Mercaptoundecanoic acid) employed for the preparation of self-assembled monolayers (SAMs).

a colorimetric assay with the chromogenic substrate nitrocefin[12] in order to quantitatively detect the catalytic activity of β-lactamase. The stability of the surface-immobilized enzyme was explored through enzymatic activity assays. The enzymatic activities for the enzyme in solution and immobilized on the different interfaces were compared, allowing us to judge and discuss the effect of enzyme surface coverage and mass transport on the catalytic activity.

II. EXPERIMENT

A. Materials

The thiol derivatives (Fig. 2) were dissolved in ethanol at a concentration of 5 mM. NeutrAvidin, purchased from Molecular Probes, Leiden, The Netherlands, was dissolved in 4-(2-Hydroxyethyl) piperazine-1-ethane-sulfonic acid (HEPES) II buffer (10 mM HEPES, 150 mM NaCl, *p*H 7.4) at a concentration of 0.15 mg/ml. Biotin-β-lactamase variants (Q269C) were produced as described before[9] and dissolved in HEPES II buffer at a concentration of 2 μM. PLL-*g*-PEG/PEG-biotin was synthesized and characterized according to published protocols.[9] PLL(20kDa)-*g*(3.4)-PEG(2kDa)/PEG(3.4kDa)-biotin was dissolved in HEPES I buffer (10 mM HEPES, *p*H 7.4) at a concentration of 1 mg/ml. DTT (Dithiothreitol) was dissolved at a concentration of 50 mM in HEPES II buffer. Human serum (10 mg, purchased from Sigma-Aldrich, Germany) was dissolved in water at a concentration of 1 mg/ml. D-biotin, purchased from Sigma-Aldrich, was dissolved in HEPES II buffer at a concentration of 1.15 mM. HELLMANEX, purchased from Hellma GmbH, Germany, was diluted with water to a concentration of 1%. Nitrocefin, purchased from Calbiochem, was dissolved in assay buffer [10 mM HEPES-NaOH, *p*H 7.4, 150 mM NaCl, 0.25 mg/ml BSA, 0.5% (v/v) dimethyl sulfoxide] at different concentrations. All other reagents were purchased from Sigma-Aldrich, Germany. Buffers were prepared using ultrapure water (18 MΩ cm).

B. Substrates

LaSFN9 slides (Schott, $n=1.85$ at $\lambda=633$ nm) were carefully cleaned with 1% HELLMANEX, ultrapure water and ethanol in turn, coated with 2 nm chromium (Cr) followed by 50 nm of gold (Au) by thermal evaporation in a commercial instrument (Edwards). For matrices C and D of Fig. 1, either 4 nm Nb_2O_5 or 10 nm SiO_2 were sputter coated on top of the Cr/Au substrate by a Leybold Z600 magnetron sputtering unit at the Paul Scherrer Institute, Villigen, Switzerland. Metal-oxide-coated substrates were cleaned in a plasma cleaner (Technics Plasma-Prozessor 200-G, Germany, 300 W) for 5 min before surface modifications using oxygen as the plasma gas. For matrices A, B and E, freshly coated Cr/Au substrates were directly immersed into the thiol solutions, then sealed, and kept overnight at room temperature. The substrates were removed, rinsed thoroughly with ethanol, and blowndried in a stream of nitrogen. The substrates were used immediately after preparation.

C. Surface modification

The experimental setup[13] allows for the simultaneous recording of the reflectivity as a function of the angle of incidence, θ. The wavelength λ of the laser light source is 632.8 nm (HeNe laser). Figure 1 shows the schematics of the five matrices studied in this work. For matrix A, a binary thiol mixture containing 10 mol % of the biotinylated thiol and 90 mol % of the OH-terminated diluent thiol was allowed to assemble at the Cr/Au surface overnight. Mixed solutions were prepared at a net thiol concentration of 357 μM in absolute ethanol (99%). The biotin-terminated self-assembled monolayers (SAMs) were used for the binding of NeutrAvidin for 30 min and the further attachment of biotin-β-lactamase for another 30 min. PLL-*g*-PEG/PEG-biotin was attached to Nb_2O_5 (matrix C), SiO_2 (matrix D), and a carboxyl-terminated SAM via electrostatic interaction (matrix B), and by covalent binding to the carboxyl-terminated SAM via N-hydroxysuccinimide (NHS) ester activation[14] (matrix E). In all these four cases, PLL-*g*-PEG/PEG-biotin was used as the basis for the specific immobilization of NeutrAvidin. The attachment of NeutrAvidin and biotin-β-lactamase for all five matrices was achieved by two different strategies:

(1) Stepwise (sequential) binding of NeutrAvidin and biotin-β-lactamase:
Chips were incubated with NeutrAvidin for 30 min and rinsed with HEPES II buffer; then the biotin-β-lactamase was injected into the flow cell and incubated for 30 min. Subsequently, unspecifically bound β-lactamase was removed by rinsing with HEPES II buffer.

(2) One-step binding of the complex of NeutrAvidin/biotin-β-lactamase preformed in solution:
NeutrAvidin was first mixed with biotin-β-lactamase in a molar ratio of 1:2 in HEPES II buffer for 30 min at room temperature and a final concentration of 0.03 mg/ml NeutrAvidin and 0.03 mg/ml biotin-β-lactamase. Subsequently, the formed complex was immobilized on the surface until equilibrium was reached (~30 min incubation). Release of immobilized β-lactamase from the surface was achieved by incubation with 50 mM DTT in HEPES II buffer for 15 min.

All measurements were done at room temperature.

D. Optical waveguide lightmode spectroscopy (OWLS) measurements

OWLS measurements were performed with chips based on matrix C. Optical waveguide chips for OWLS measurements were purchased from Microvacuum, Ltd. (Budapest, Hungary), and consisted of a 1-mm-thick AF45 glass substrate and a 200-nm-thick $Si_{0.75}Ti_{0.25}O_2$ waveguiding surface layer. A 12-nm-thick Nb_2O_5 layer was sputter coated on top of the waveguiding layer. Nb_2O_5-coated waveguide chips were sonicated in 0.1 M HCl for 10 min, thoroughly rinsed with ultrapure water, dried in a stream of nitrogen, and treated for 2 min in an oxygen plasma in a plasma cleaner/sterilizer PDC-32G (Harrick, Ossining, NY). After inserting the chips into the OWLS flow cell, PLL-*g*-PEG/PEG-biotin was immobilized on the surface as a platform for the further attachment of NeutrAvidin and biotin-β-lactamase. All steps were performed according to the pre-incubation protocol described above.

E. Regeneration of binding matrices

The feasibility to regenerate the chip surfaces was tested for all five matrices; 0.1 M HCl was used to regenerate the substrates for matrices A, B and E, while for matrices C and D, we used 1% HELLMANEX. The same binding procedures for PLL-*g*-PEG/PEG-biotin, NeutrAvidin, and biotin-β-lactamase were used based on the stepwise binding strategy to compare the binding abilities after regeneration with those before regeneration.

F. Enzyme activity assay

The activity of β-lactamase was measured quantitatively through the reaction with the chromogenic substrate nitrocefin, which undergoes a distinctive color change from yellow (λ_{max}=390 nm at *p*H 7.4) to red (λ_{max}=486 nm at *p*H 7.4) as the amide bond in the β-lactam ring is hydrolyzed by the β-lactamase. The absorbance maximum of the nitrocefin product shifted from λ=486 to λ=500 nm in the presence of bovine serum albumin (BSA; 0.25 mg/ml), which proved to be necessary for the stabilization of low concentrations of β-lactamase in solution.

Chips with surface-immobilized biotin-β-lactamase based on pre-incubation strategies were rinsed in HEPES II buffer with a flow rate of 36.5 μl/min for 40 h at room temperature before starting the enzymatic activity assay, in order to remove all nonspecifically bound enzymes from the sensor surface. The combination of SPR and FOAS[2] (Fig. 3) was used for the assay. The basic element of the FOAS sensor 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, and to the two optical fibers for excitation and detection, respectively. The setup shown in Fig. 3 consists of a 7 W tungsten halogen light source (HL-2000-LL) driven by a power supply, a "Z" configuration "FIA-Z-SMA" flow cell to measure the optical absorbance of fluids moving through the flow injection systems, a S2000 miniature fiber optic spectrometer

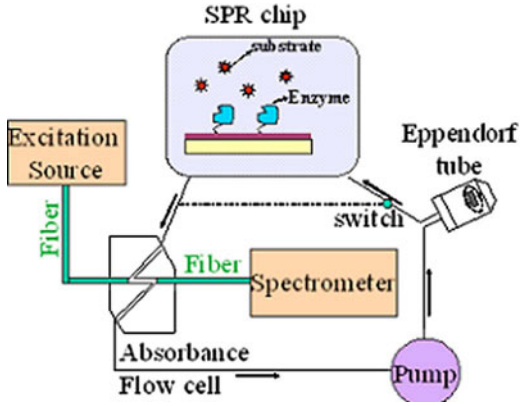

FIG. 3. Schematic of the FOAS setup for the enzymatic activity assay.

with its own operating software, and a couple of 2-m-long, 400-μm-diam optical fibers (P400-2-UV/VIS), which are used for connecting the light source to the flow cell, and the flow cell to the spectrometer, respectively. In the flow cell, the optical fibers were connected to SMA fittings to project and receive light through the central axis of the Z structure. The flow cell has a 10 mm optical path length and a 1.5 mm inner diameter, and uses UV-visible silica windows as wetting surfaces at each fiber optic junction. All components described above were purchased from Ocean Optics, Inc., The Netherlands. A peristaltic pump was used to circulate the substrate solution from an Eppendorf tube stirred by a magnetic stirring bar to the FOAS and SPR flow cell. The whole setup was isolated from ambient light.

In the enzymatic assay, first the dark (background) signal was determined. Then, assay buffer was injected into the flow cell serving as the reference, followed by rinsing the nitrocefin solution through the FOAS system for several minutes in order to establish a base line, before the solution was allowed to run through the SPR flow cell. The substrate changed to product by the catalytic action of biotin-β-lactamase immobilized on the SPR sensor surface. The change in the absorbance at 500 nm was recorded as a function of time. In order to test the desorption and contamination of enzymes in the system during the assay process, we short cut the connection between SPR flow cell and FOAS system for several minutes, and then let the solution run through the SPR flow cell again.

The enzyme stability is usually the critical factor that determines the lifetime of an enzyme-based biosensor. In order to demonstrate the stability of β-lactamase attached to the sensor quantitatively, we performed the enzymatic activity assay with SPR chip based on matrix A onto which β-lactamase was freshly bound, followed by a short rinse in HEPES II buffer instead of the 40 h rinsing. We also let the substrate solution run across a NeutrAvidin surface with no β-lactamase immobilized, as a background control of the turnover of the nitrocefin. The kinetics of the absorbance increase for (a) the background, (b) freshly bound

β-lactamase and (c) after 40 h incubation was compared for chips based on matrix A and using 80 μM nitrocefin solution with the flow rate of 1.0 ml/min.

The activity of biotin-β-lactamase was also tested in solution utilizing the FOAS system, the results serving later as controls for the evaluation of the kinetic activity of the surface-immobilized enzymes. For the activity tests in solution, we added 0.3 nM β-lactamase to different concentrations of nitrocefin solutions (8, 12, 20, 28, 40, 70, 80, 120, 160, and 200 μM) in assay buffer. The mixture was then immediately circulated through the FOAS flow cell while recording the absorbance increase at λ=500 nm. The flow rate of 1.0 ml/min was applied to all the measurements described above.

A set of kinetic runs of β-lactamase was recorded using different concentrations of nitrocefin at room temperature. The initial reaction velocities of the change in absorbance were collected for the Michaelis–Menten analysis. Data were fitted according to the equation

$$v = \frac{V_{\max}[S]}{[S] + K_M},$$

where v is the observed initial reaction velocity, $V_{\max}$ is the maximal velocity, $[S]$ is the substrate concentration, and K_M represents the Michaelis constant. The higher the K_M value, the lower the enzyme-substrate affinities. K_M and $V_{\max}$ values for an enzyme are determined by fitting data at various substrate (nitrocefin) concentrations; k_{cat} (turnover number) is another important parameter that determines the catalytic efficiency of an enzyme, and is defined as

$$k_{\text{cat}} = \frac{V_{\max}}{[E_{\text{total}}]}.$$

This quantity represents the number of moles of substrate converted to product per second and mole of enzyme.

III. RESULTS AND DISCUSSION

A. Interfacial architectures

Five different interfacial architectures were produced and served as sensor platforms based on the specific attachment of biotin-functionalized β-lactamase (biotin-β-lactamase). Biotinylated molecular systems were used resulting in five generic biotin-NeutrAvidin binding matrices. As shown in Fig. 1, specific thiols (cf. Fig. 2) were assembled onto chromium/gold films from ethanolic solution for matrices A, B and E. The functionalization of matrix A was achieved by forming biotinylated self-assembled monolayers (SAMs) with a binary mixture of biotin-terminated thiols and suitable diluent thiols,[15] followed by the binding of NeutrAvidin. The biotin-β-lactamase utilized in this study contains a single biotin group and a 2.9-nm-long DTT-cleavable disulfide bridge linker between the biotin group and the surface exposed cystein residue of the genetically modified lactamase. Release of β-lactamase from the surface by DTT was quantitative, thus proving that all β-lactamase molecules were specifically bound to the surface via their biotin groups.[16]

For matrices B and E, carboxyl-terminated alkanethiol SAMs were used for the immobilization of the graft copolymer PLL-*g*-PEG/PEG-biotin via either electrostatic interactions or covalent binding. Nb_2O_5 and SiO_2 were sputter coated on top of the Cr/Au substrate for matrix C and D. Nb_2O_5 and SiO_2-coated chips were used in view of the high negative surface charge density at neutral *p*H and the resulting strong electrostatic binding of the polycationic polymers PLL-*g*-PEG/PEG-biotin. The general functionality of this type of interface has already been successfully demonstrated[4] for applications in bioaffinity sensing. PLL-*g*-PEG/PEG-biotin has been chosen as the surface immobilization platform for four out of the five matrices. At neutral *p*H, PLL-*g*-PEG/PEG-biotin spontaneously and firmly attaches from aqueous solutions to negatively charged surfaces through multiple-site electrostatic (matrix B, C, and D) or covalent (matrix E) interactions between the polycationic PLL backbone and the negatively charged surface.[17,18] The high density of PEG molecules results in a brush-like, flexible, uncharged, strongly hydrated, and noninteractive interface, which allows for gentle immobilization of proteins and other biomolecules. The biologically active tertiary structure of the attached protein is preserved as well as the accessibility of its active site to the substrate reaction partner. NeutrAvidin can specifically bind to the biotin groups of the polymer, and the immobilization of β-lactamase onto the NeutrAvidin occurs through a biotinylated spacer.

The SPR technique allows us to determine *in situ* the amount of biotin-β-lactamase as well as PLL-*g*-PEG/PEG-biotin and NeutrAvidin bound to the surface. A full angular scan was recorded for every single layer after surface attachment. All respective angular shifts were Fresnel analyzed. From Fresnel's equations, one can calculate the optical thickness of the coating (assuming for PLL-*g*-PEG/PEG-biotin and biotin-β-lactamase, a refractive index of $n=1.41$, and for NeutrAvidin, a refractive index of $n=1.45$).[14]

B. Comparison of different matrices

PLL-*g*-PEG/PEG-biotin monolayers form a soft, gel-like matrix with substantial flexibility of the PEG chains (and the biotin group attached to the PEG termini). A consequence is that after the initial binding of NeutrAvidin to the PEG-biotin surface, PEG chains (including both neighboring PEG chains planted on the same PLL backbone and those from other PLL-*g*-PEG/PEG-biotin chains) with free biotin groups may additionally occupy biotin-binding sites of the surface-bound NeutrAvidin. This time-dependent process may finally result in the majority of the originally free binding sites of NeutrAvidin being blocked, and therefore no longer available for subsequent binding of biotinylated molecules[4] in this stepwise functionalization protocol. The alternative to get a higher amount of surface-bound biotin-β-lactamase is to preincubate the mixture of NeutrAvidin and biotin-β-lactamase, and subsequently attach the complex to the surface-bound PLL-*g*-PEG/PEG-biotin in one step. DTT (Dithiothreitol) was used to cleave the disulfide linkage between β-lactamase

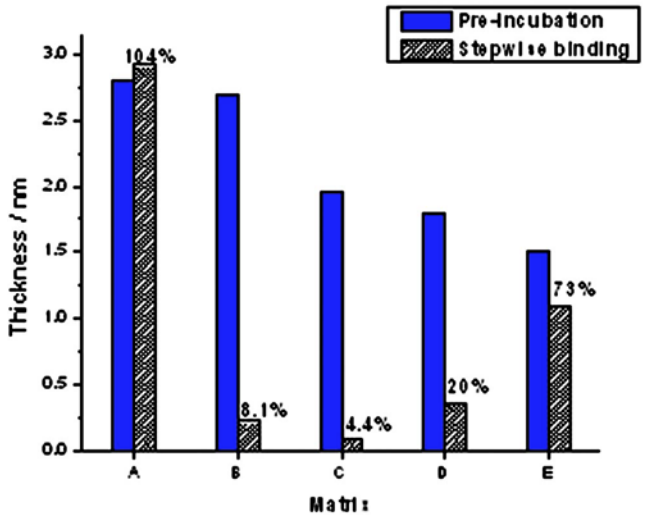

FIG. 4. Comparison of the optical thickness of surface bound β-lactamase between stepwise binding and pre-incubation strategies based on five different kinds of matrices detected by SPR. Inset numbers indicate the values of $Th_{\text{stepwise}}/Th_{\text{pre-incubation}}$ (ratio of optical thickness of surface attached biotin-β-lactamase based on stepwise binding strategy to the obtained thickness based on pre-incubation strategy. A, B, C, D, and E: symbols of matrices used in the study.

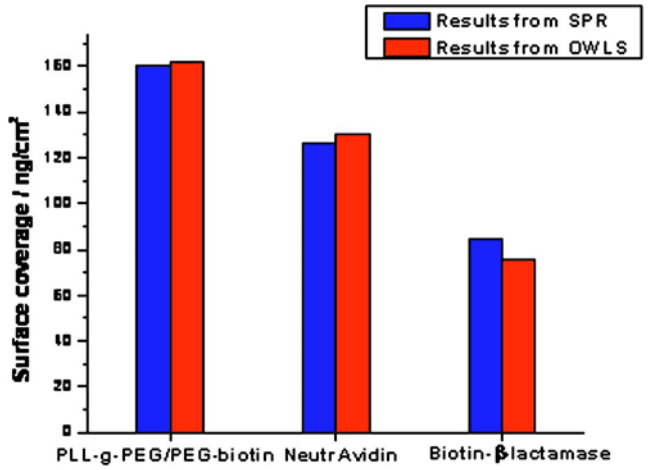

FIG. 5. Comparisons of the surface coverage amounts of PLL-*g*-PEG/PEG-biotin, NeutrAvidin, and β-lactamase based on matrix C with pre-incubation strategy obtained from SPR and OWLS measurements, respectively.

and its biotin label. This step allows us to compare quantitatively the amount of surface-bound NeutrAvidin/biotin-β-lactamase complex with the mass of β-lactamase released upon DTT exposure. We refer to this strategy as the pre-incubation protocol.

The results of the comparative study of stepwise and pre-incubation protocols for all five matrices are given in Fig. 4. Comparable amounts of immobilized enzyme were obtained for matrix A with both protocols, while for matrices B, C, and D, a significantly lower surface coverage of biotin-β-lactamase was found for the stepwise binding compared to the pre-incubation protocol. These differences can be understood on the basis that matrix A is a rather stiff, two-dimensional molecularly ordered surface-immobilization platform, for which additional blocking of biotin-binding sites of NeutrAvidin (as discussed above) is impossible or very unlikely, while matrices B, C, and D, prepared with the brush-like, flexible polymer, PLL-*g*-PEG/PEG-biotin, are rather soft matrices for the surface attachment and prone to major rearrangements of the immobilized molecules. The property of matrix E assembled based on the covalent binding strategy of PLL-*g*-PEG/PEG-biotin with the carboxyl-terminated SAMs is expected to be intermediate in view of the lower surface coverage of PLL-*g*-PEG/PEG-biotin, which subsequently results in a lower amount of bound biotin-β-lactamase compared to other PLL-*g*-PEG/PEG-biotin based matrices (shown in Fig. 4), especially to matrix B. The low surface coverage of PLL-*g*-PEG/PEG-biotin effectively reduced the NeutrAvidin-blocking effect by PEG groups planted on different PLL backbones.

C. Comparison of different optical detection methods (SPR vs OWLS)

Figure 5 shows the surface coverage, determined by SPR and OWLS, respectively, of PLL-*g*-PEG/PEG-biotin, NeutrAvidin, and β-lactamase on matrix C prepared by the pre-incubation protocol. OWLS is another *in situ*, label-free, evanescent-field-based technique that measures the refractive index and thickness of adsorbed macromolecules.[19] The typical detection sensitivity of the instrument is 1 ng/cm^2. The same protocols as those for SPR measurements were applied for the *in situ* investigation of the surface adsorption and immobilization processes utilizing Nb_2O_5 as the substrate (matrix C).

With respect to the data analysis of SPR and OWLS, the adsorbed mass of polymer and protein was calculated from the measured effective refractive indices and thickness values according to

$$A = d\frac{(n - n_o)}{\partial n/\partial c}$$

and the formula of de Feijter *et al.*[20] With n_0 being the refractive index of the solution, the adsorbed amount A of protein per unit area can be calculated with the refractive index increment ∂_n/∂_c of the protein solution, which is determined independently.[9,15] $\partial_n/\partial_c = 0.212$ ml/g for NeutrAvidin, 0.18 ml/g for β-lactamase, and 0.16 ml/g for polymers were used for the calculation.[14]

With deviations of less than 5% in the adsorbed mass on surfaces of type matrix C (Fig. 5), we conclude that the results of the two independent sensing techniques are in excellent agreement.

D. Estimation of regeneration possibilities

Regeneration procedures are able to disassemble a supramolecular architecture of a sensor chip into its individual building blocks followed by a reassembly process, thus al-

TABLE I. Evaluation of regeneration possibilities for five matrices based on stepwise binding strategy. Numbers represent the surface coverages of PLL-*g*-PEG/PEG-biotin, NeutrAvidin and biotin-β-lactamase after chip regeneration, expressed as percentage of the original coverage (i.e., before regeneration).

Matrix	A	B	C	D	E
	Biotin-terminated SAM	Carboxyl-terminated SAM	Nb_2O_5	SiO_2	Carboxyl-terminated SAM (NHS ester)
Regeneration possibility	[c]N*	Poor	Y[a]	N[b]	N[b]
PLL-*g*-PEG/PEG-biotin	···	105%	90%	···	···
NeutrAvidin	···	54%	98%	···	···
Biotin-β-lactamase	···	···	74%	···	···

[a]Y: Surface can be regenerated.
[b]N: Surface cannot be regenerated.
[c]N*: By using desthio- or imido-biotin derivatized thiols for the SAM formation, regeneration would be possible (see Ref. 15)

lowing for the repetition of surface functionalization and reuse as sensor. Table I shows the regeneration results for all five matrices. The numbers indicate the ratio of the amount of bound PLL-*g*-PEG/PEG-biotin, NeutrAvidin and biotin-β-lactamase after regeneration to that before regeneration (for procedures: see Sec. II C). Matrix C on the Nb_2O_5 surface can be regenerated at a level of 74% (in terms of restored enzyme coverage) using 1% HELLMANEX to deionize the metal oxide surface and release the electrostatically attached PLL-*g*-PEG/PEG-biotin. A new supramolecular architecture can be formed if the surface is again exposed to a fresh polymer solution. The amounts of reassembled PLL-*g*-PEG/PEG-biotin, NeutrAvidin, and biotin-β-lactamase on matrix C show a satisfactory regeneration. Matrix B, built on the carboxyl terminated SAM via electrostatic interactions, can be partly regenerated to 54% of NeutrAvidin rebound, indicating the lower amount of reassembled PLL-*g*-PEG/PEG-biotin. However, hardly any signal of biotin-β-lactamase attachment can be obtained during the reassembled process derived from the SPR data simulation. Assuming that a fraction of biotin-β-lactamase might bind to the surface-bound NeutrAvidin, there must be some loosely bound molecules (i.e., PLL-*g*-PEG/PEG-biotin or NeutrAvidin attached to PLL-*g*-PEG/PEG-biotin) desorbing upon rinsing, indicating the unstable property of Matrix B after regeneration. TheSiO_2 substrate of Matrix D is so brittle that the applied regeneration process totally disturbed the shape of the SPR curve (details not shown), indicating a major perturbation of the sensor surface. As a result, matrix D cannot be regenerated in spite of its assembly mechanism via electrostatic interaction. Matrix E was assembled through covalent attachment. It cannot be regenerated by simply deprotonating the SAM surface. With respect to matrix A, NeutrAvidin binds biotin at four binding sites, with an extremely high binding constant ($K_d = 10^{13-15}$ M^{-1}), which makes the binding process effectively irreversible. However, the NeutrAvidin-biotin system of matrix A offers a regeneration strategy by using ligands of biotin analogues with a reduced affinity for the binding pocket. This option could result in a design strategy for the competitive replacement of one ligand by another one with a higher binding constant thus making the sensor surface reusable.[15,21,22]

E. Enzymatic activity assay

Enzyme stability, both in terms of active conformation and stable immobilization, is a key factor for achieving high sensitivity and extended lifetime of enzyme-based biosensors. In the standard enzyme assay protocols used in this study, the SPR chip surfaces were either tested immediately after enzyme immobilization (based on pre-incubation strategies), or first rinsed constantly for 40 h with pure buffer before the enzymatic assay was performed in order to remove any loosely bound β-lactamase. Turnover kinetics in 80 μM nitrocefin solution were monitored as a function of time, and the data compared for the case of the mere background (on a NeutrAvidin surface with no β-lactamase immobilized, negative control), and for chips (matrix A) with freshly bound β-lactamase, and after 40 h of rinsing (see Sec. II E). The enzymatic turnover after the exposure of the freshly bound enzyme-coated chip to the substrate solution is shown in Fig. 6. The immobilized biotin-β-lactamase showed significant enzymatic activity. Upon short cutting the connection between the SPR and the FOAS flow cell (cf. Fig. 3), the enzymatic turnover of nitrocefin did not cease as

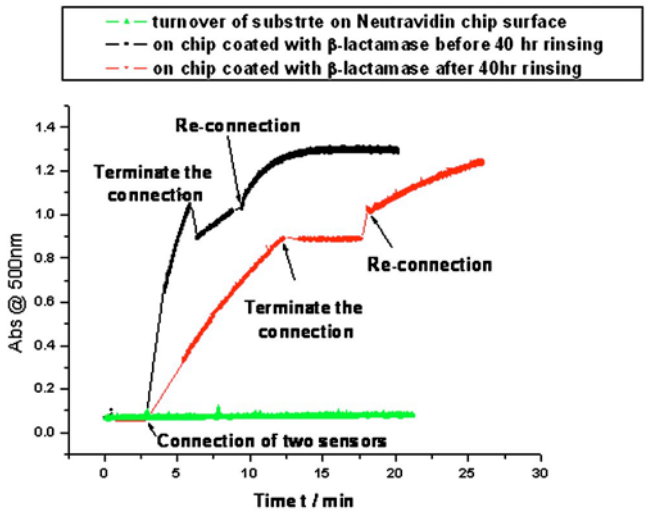

FIG. 6. Enzymatic activity and stability of surface immobilized β-lactamase based on matrix A.

TABLE II. SPR results and kinetic parameters obtained from miniature fiber optic absorbance spectroscopy (FOAS) for surface immobilized (based on pre-incubation strategies on matrices B-E, see Fig. 2) and free β-lactamase in solution. The numbers represent mean values ± standard deviation. PLL-PEG/PEG-biotin polymer and enzyme surface coverages on chip were obtained from SPR simulation. C, the mole concentration of β-lactamase involved in the catalysis of substrate inside the SPR flow cell. K_M, Michaelis constant, the substrate concentration at which the reaction velocity is half-maximal; k_{cat}, turnover number, the number of reaction events per enzyme molecule and second; and k_{cat}/K_M, catalytic efficiency of the enzyme.

	Type of interfacial matrix				
	(B) Carboxyl terminated SAM	(C) Nb_2O_5	(D) SiO_2	(E) Carboxyl terminated SAM (NHS ester)	"Free" enzyme in solution
PLL-PEG/PEG-biotin (ng/cm^2)	168	160	96	63	
Enzyme surface coverage (ng/cm^2)	52.0	42.4	19.3	9.0	
C (n mol L^{-1})	5.17	4.27	1.94	0.91	
K_M (10^{-6} mol L^{-1})	820±270	730±90	560±110	500±130	200±20
k_{cat} (s^{-1})	187±53	212±22	374±59	533±108	814±71
k_{cat}/K_M (10^6 mol^{-1} L s^{-1})	0.23±0.07	0.29±0.03	0.67±0.11	1.07±0.21	4.07±0.36

expected but continued at a lower rate, due to loosely bound enzyme molecules that desorbed from the SPR chip during the assay and catalyzed the turnover of the substrate in solution. If the two systems were reconnected, the higher enzymatic turnover was reestablished.

Figure 6 shows another similar experiment, except that this time the chip was rinsed in the SPR flow cell with pure buffer for 40 h and at a low flow rate of 37 μl/min. The chip showed again significant enzymatic activity, however, with a lower reaction rate this time in comparison to the freshly coated β-lactamase chip. Since reduced surface coverage of enzyme based on matrix A resulted in a lower initial reaction velocity,.[2] this is obviously a consequence of the desorption of loosely bound adsorbed β-lactamase during the long-term buffer rinse, which is supported by our previous study with OWLS detection.[9] If the connection between the SPR and the FOAS flow cell was short cut, the turnover of nitrocefin ceased completely, indicating that this time negligible amounts of enzyme were present in solution and that the 40 h buffer rinsing had efficiently removed all loosely bound enzymes. Combined with the observation that no turnover of nitrocefin was observed if the substrate solution flowed across a chip surface that had immobilized NeutrAvidin, but no β-lactamase (cf. Fig. 6), we therefore conclude that the long-term exposure to buffer had indeed removed all or most of the unspecifically bound β-lactamase molecules. The enzyme, specifically immobilized on the chip, was found to be stable and unable to be desorbed from the SPR chip during the assay.

F. Michaelis–Menten analysis

Kinetic parameters were analyzed for the activity of β-lactamase immobilized on surface. The activity of the enzyme was also tested in solution using the FOAS system, the results serving later as controls for the evaluation of the kinetic activity of the surface-immobilized enzymes. A set of kinetic runs for a Michaelis–Menten analysis of β-lactamase was performed, again with the substrate nitrocefin.[9] Values of the kinetic parameters K_M and k_{cat} determined by measuring initial reaction velocities at various substrate concentrations are summarized in Table II. The relatively high standard deviations are due to the limited nitrocefin concentration range accessible in the Michaelis–Menten experiment as a result of the limited nitrocefin solubility. In order to determine the catalytic efficiency, k_{cat}/K_M, of an immobilized enzyme, the exact amount of the surface immobilized protein has to be known. For this purpose, SPR was utilized as an independent technique for the quantitative determination of the interfacial enzyme density. Table II shows the corresponding data of β-lactamase. From the Fresnel analysis, one can calculate the optical thickness of the biotin-β-lactamase layer (remaining molecules after 40 h rinsing process), and from that the adsorbed mass.

The effect of β-lactamase surface immobilization via matrix A (two-dimentional matrix) on the catalytic activity of the enzyme has been discussed in detail in Ref. 2. In this work, comparison is made between matrices B, C, D, and E (three-dimentional matrices), which were assembled on the basis of PLL-*g*-PEG/PEG-biotin. Results listed in Table II show that the K_M values, which indicate the enzyme-substrate affinities, were all higher than that of the "free" enzyme in solution. Together with the observed lower k_{cat} and k_{cat}/K_M values, corresponding to the turnover number and enzyme efficiency, respectively, we conclude that enzymatic activity for surface-immobilized β-lactamase is reduced compared to free enzymes in solution. Furthermore, a gradual reduction in the K_M and increase in k_{cat}/K_M values were observed with decreasing enzyme surface coverage. The decreased protein surface coverage is believed to minimize the steric crowding effect of the surface-bound enzyme molecules, and to accelerate the recognition process between enzyme and substrate. This is consistent with our previous work with respect to comparison of catalytic activity of β-lactamase based on matrix A with different enzyme sur-

face coverages.[2] A lower k_{cat} value, which indicates the ability of product formation and release from the active site, was found to be accompanied by a higher surface coverage of the PLL-*g*-PEG/PEG-biotin polymer and enzyme (cf. Table II). However, our previous results based on matrix A show a different trend of k_{cat} value (k_{cat} values of surface immobilized enzymes with different binding densities are quite similar).[2] The separate processes of enzyme catalysis within the SPR flow cell and the absorbance detection in the FOAS flow cell confirmed that mass transport plays a very important role in the observation of substrate turnover. This was supported by the results that higher working flow rate generated by the peristaltic pump increased the initial reaction velocities on matrix B as judged by the change in absorbance (detail not shown). Different from the two-dimensional planar system of matrix A, the quasi-three-dimensional architecture of the PLL-*g*-PEG/PEG-biotin polymer will largely influence the mass transport process of the product. Higher density of polymer contributes to a more complex three-dimensional system, resulting in lower mass transport rates and k_{cat} values.

IV. CONCLUSIONS

In this paper, attempts to control the density and presentation of biotin-β-lactamase at the solid-liquid interface have been undertaken through various strategies for site-specific immobilization and different detection methods. Our results show that all five matrices are promising for the design of bioaffinity sensors, although quantitative differences were found, as an evidence for the distinct stiffness of different matrices. With regard to the enzymatic activity of biotin-β-lactamase, the effects of steric crowding of surface-bound enzymes and mass transport process may influence the kinetic parameters of catalytic analysis. The systematic study of the influencing factors enabled us to better understand the molecular mechanism underlying the substrate-enzyme interaction with different interfacial architectures.

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[1] R. L. Rich and D. G. Myszka, J. Mol. Recognit. **16**, 351 (2003).
[2] F. Xu, G. Zhen, F. Yu, E. Kuennemann, M. Textor, and W. Knoll, J. Am. Chem. Soc. **127**, 13084 (2005).
[3] J. Homola, Anal. Bioanal. Chem. **377**, 528 (2003).
[4] N. Huang, J. Vörös, S. M. De Paul, M. Textor, and N. D. Spencer, Langmuir **18**, 220 (2002).
[5] K. D. Park and S. M. Kim, *Poly (Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, edited by J. M. Harris (Plenum, New York, 1992), pp. 283–301.
[6] M. D. Marazuela and M. C. Moreno-Bondi, Anal. Bioanal. Chem. **372**, 664 (2002).
[7] J. R. Knowles, Acc. Chem. Res. **18**, 97 (1985).
[8] W. J. Albery and J. R. Knowles, Biochemistry **15**, 5588 (1976).
[9] G. Zhen, V. Eggli, J. Vörös, P. Zammaretti, M. Textor, R. Glockshuber, and E. Kuennemann, Langmuir **20**, 10464 (2004).
[10] D. M. Livermore, Clin. Microbiol. Rev. **8**, 557 (1995).
[11] W. Huang, J. Wang, D. Bhattacharyya, and L. G. Bachas, Anal. Chem. **69**, 4601 (1997).
[12] C. H. Ocallagh, A. H. Shingler, S. M. Kirby, and A. Morris, Antimicrob. Agents Chemother. **1**, 283 (1972).
[13] T. Liebermann and W. Knoll, Colloids Surf., A **171**, 115 (2000).
[14] J. G. Gordon and J. D. Swalen, Opt. Commun. **22**, 374 (1977).
[15] W. Knoll, M. Zizlsperger, T. Liebermann, S. Arnold, A. Badia, M. Liley, D. Piscevic, F. J. Schmitt, and J. Spinke, Colloids Surf., A **161**, 115 (2000).
[16] http://www.piercenet.com
[17] G. L. Kenausis, J. Vörös, D. L. Elbert, N. Huang, R. Hofer, L. Ruiz-Taylor, M. Textor, J. A. Hubbell, and N. D. Spencer, J. Phys. Chem. B **104**, 3298 (2000).
[18] N. Huang, R. Michel, J. Vörös, M. Textor, R. Hofer, A. Rossi, D. L. Elbert, J. A. Hubbell, and N. D. Spencer, Langmuir **17**, 489 (2001).
[19] J. Vörös, J. J. Ramsden, G. Csucs, I. Szendro, S. M. De Paul, M. Textor, and N. D. Spencer, Biomaterials **23**, 3699 (2002).
[20] J. A. de Feijter, J. Benjamins, and F. A. Veer, Biopolymers **17**, 1759 (1978).
[21] L. S. Jung, K. E. Nelson, C. T. Campbell, P. S. Stayton, S. S. Yee, V. Perez-Luna, and G. P. Lopez, Sens. Actuators B **54**, 137 (1999).
[22] W. Knoll, L. Angermaier, G. Batz, T. Fritz, S. Fujisawa, T. Furuno, H. J. Guder, M. Hara, M. Liley, K. Niki, and J. Spinke, Synth. Met. **61**, 5 (1993).