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Optical Biosensors. Monitoring Studies of Glycopeptide Antibiotic Fermentation Using White Light Interference

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This paper describes the design, characterization, and use of an optical biosensor suited for the process control of biotechnological processes. The detector principle is based on reflectometric interference spectroscopy (RIfS). RIfS enables a label-free, product-specific monitoring, with a future outline for on-line process control. The potential of the RIfS biosensor is exemplified by the qualitative and quantitative monitoring of the microbial production of vancomycin-type glycopeptide antibiotics.

Many pharmaceutical compounds that are not available by chemical synthesis in reasonable amounts are produced by fermentation. Antibiotics, proteins, and food and feeding supplements are produced by bioengineered organisms in large-scale fermentors. These processes are controlled by measuring chemical and physical parameters during the course of the production, which are pH, temperature, optical density, and dissolved oxygen.[1] However, these system-descriptive parameters lack in most cases qualitative and quantitative information about the desired compound itself. The determination of the state of the fermentation with regard to concentration of substrates or products is, with the exception of few in situ sensors, e.g., for glucose,[2] usually performed by the use of HPLC[3] with expensive multichannel devices.[1] However, specific biosensors provide an alternative analytical approach, since they integrate both an on-line, qualitative and a quantitative response toward the compound of interest.

In the present work, we designed and developed a biosensor based on the reflectometric interference spectroscopy (RIfS). Originally, RIfS was established for the detection of biomolecular interactions[4] and applied for small-molecule detection by chemical sensor surfaces.[5−7] We transferred this concept to the production control of fermentor samples, exemplified with glycopeptide antibiotics vancomyin and balhimycin.[8] Vancomycin represents an antibiotic of the last resort in the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* strains (MRSA) and has a market share close to 1 billion U.S. dollars.[9] Glycopeptide antibiotics inhibit the cell wall biosynthesis in Gram-positive bacteria by specific binding to the D-Ala-D-Ala-termini of peptidoglycan precursors.[10] This specific interaction is the principle made use of for the presented biosensor as a typical example for biospecific detection.

EXPERIMENTAL SECTION

Chemicals. All chemicals were of analytical grade. Vancomycin hydrochloride was purchased from Fluka (Neu-Ulm, Germany). Glycopeptide antibiotic balhimycin was received from Aventis Pharma (Frankfurt, Germany). Diaminopoly(ethylene glycol) (DAPEG, mean molecular mass 2000 Da) was from Rapp Polymere (Tübingen, Germany). Aminodextran 260/10 (AMD) was prepared according to Piehler et al.[11] Chicken ovalbumin was from Sigma (Deisendorf, Germany). Protected amino acids were from NovaBiochem (Bad Soden, Germany). Trityl chloride−polystyrene resin was from Pepchem (Tübingen, Germany).

Peptide Synthesis. N^{α}-Acetylated L-Lys-D-Ala-D-Ala [Ac-Lys-$(D\text{-Ala})_2$] was synthesized on trityl chloride-resin (TCP-resin) using Fmoc/tBu strategy according to common guidelines of solid-phase

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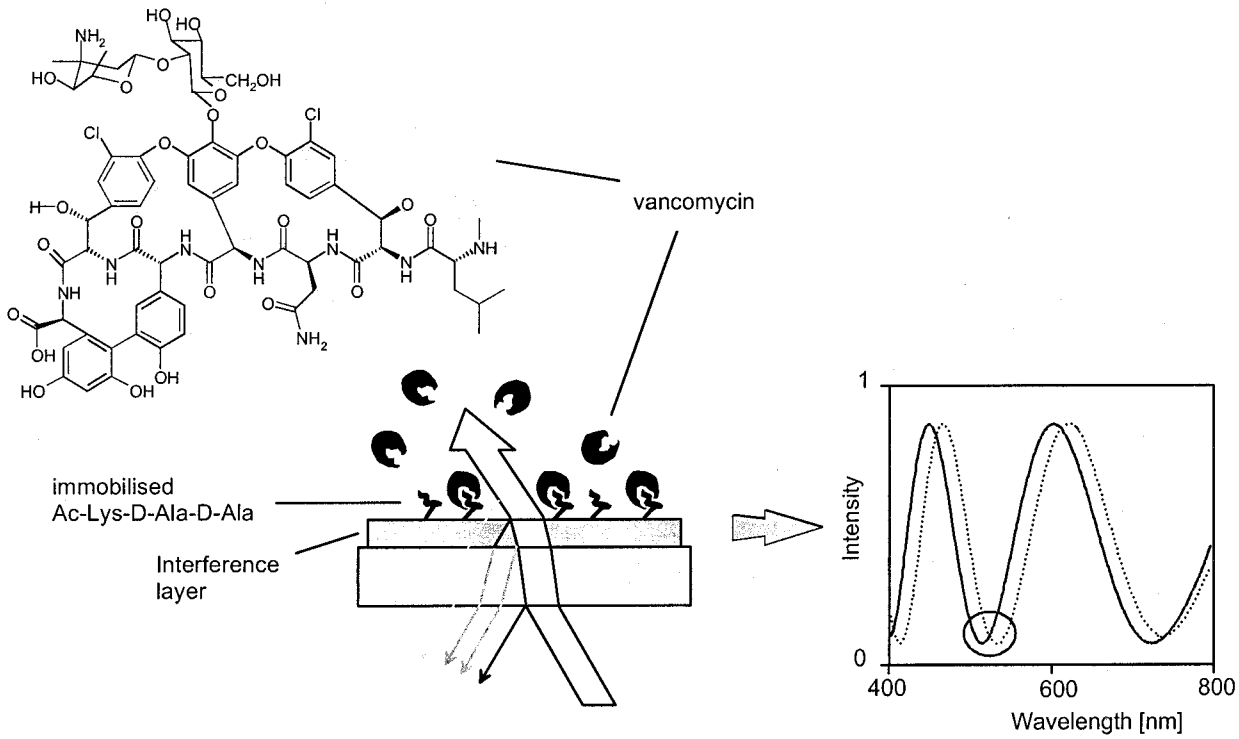

Figure 1. Principle of the reflectometric interference spectroscopy (RIfS). A thin silica layer on a glass chip is illuminated from the backside using white light. Light beams are reflected at the different layers and superimpose to form a characteristic interference pattern. Changes in the thickness the transducer surface caused by biomolecular interactions lead to a shift of the interference pattern which can be evaluated in real time.

peptide synthesis.[12] All peptides were purified by preparative HPLC and analyzed by HPLC and ES-MS.[13]

Bacteria and Fermentation Medium. The producing strain of balhimycin was *Amycolatopsis mediterranei* DSM 5908. The seed medium consisted of glucose 1.5 g/L, soybean flour 1.5 g/L, cornsteep 0.5 g/L, $CaCO_3$ 0.2 g/L, and NaCl 0.5 g/L.

Fermentations were carried out in 1-L Erlenmeyer flasks on a rotary shaker at 120 rpm and 27 °C using a production medium that consisted of glycerol 20 g/L, glucose 5 g/L, soybean peptone 10 g/L, yeast extract 3 g/L, and $CaCO_3$ 3 g/L. Samples (5 mL) were taken from the growing culture at periodical times. After centrifugation at 10.000 rpm for 10 min at room temperature, the supernatand was collected and stored at −18 C. Before analysis, the fermentor samples were thawed at room temperature.

Reflectometric Interference Spectroscopy. RIfS (Figure 1) is well described in the literature. For a detailed description and the experimental setup, see refs 14 and 15. Sample handling was performed with a flow injection analysis (FIA) system. The system was flushed with PBS buffer. Then an ovalbumin solution and the solution of the antibiotic, respectively, were loaded into the sample loop and retrograde injected with 80 μL/min. Complete regeneration of the transducer was performed with HCl (pH 1.5). All measurements were repeated three times.

Transducer Preparation. As transducer slides we used 1-mm-diameter 263 glass with layers of 10 nm of Ta_2O_5 and 330 nm of SiO_2 (Schott). These interference chips were cleaned using freshly prepared mixture of H_2SO_4/H_2O_2 (3:2, v/v), thoroughly rinsed with water and dried. The cleaned chips were silanized with glycidoxypropyltrimethoxysilane (GOPTS) for 1 h. After being washed with dry acetone and dried in a nitrogen stream, the transducers were immediately coupled with either AMD or DAPEG as described by Piehler et al.[16] To provide a carboxy-functionalized surface, the transducer was exposed to a solution of glutaric anhydride (2 mg/mL), representing the linker for N^{α}-acetylated-L-Lys-D-Ala-D-Ala peptide. The peptide was covalently attached to the surface by a peptide-coupling procedure using TBTU via the free ϵ-amino group of the lysine residue. The resulting peptide-functionalized sensor surface is shown in Figure 2.

HPLC−MS. For LC−MS couplings, a double-syringe pump (Applied Biosystems, model 140A) was used. Separations were performed on a Nucleosil C-18 2 × 100 mm, 5-μm column (Grom, Herrenberg, Germany) with a flow rate of 200 μL/min. The UV detector (UVIS 204, Linear, Reno, NV) was connected in series with the mass spectrometer.

For high-throughput HPLC−MS analyses,[13,17] the injection valve of the autosampler (model 231, Dilutor 401, Gilson, Villiers le Bel, France) was connected to the HPLC column. For sample

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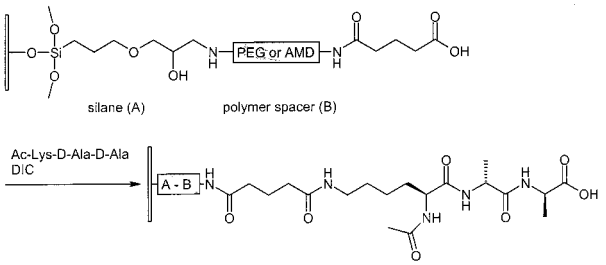

Figure 2. Peptide functionalization of the transducer surface using spacer molecules and the N^{α}-acetylated tripeptide Ac-L-Lys-D-Ala-D-Ala linked via its ϵ-amino group.

analyses, a linear gradient (0% B to 40% B in 13 min; solvent A, 0,1% TFA in water; solvent B, 0,1% TFA in acetonitrile) was used.

The MS detection was performed on a Perkin-Elmer API III TAGA Sciex triple quadrupole mass spectrometer (Thornhill, ON, Canada) equipped with an electrospray ionization source. The LC mobile-phase flow rate was reduced to 70 μL/min by means of a splitter prior to the ES interface.

To quantify glycopeptide antibiotics from standard solutions and fermentor samples, the $[M + H]^+$ pseudomolecular ions were recorded in the positive ion mode (electrospray needle voltage, 4.2 kV; orifice voltage, 100 V) by single ion monitoring (SIM) using characteristic fragment losses. The pseudomolecular ions were fragmented with argon in the collision cell and recorded by a characteristic mass loss of the vancosamine sugar moiety (Δm = 166.17 Da) or the glucose moiety (Δm = 162.15 Da) for each compound.

For recording of calibration curves, stock standard solutions of balhimycin were prepared by dissolving appropriate amounts of antibiotic in water to give a final concentration of 1 mg/mL. This solution was then successively diluted with water in concentrations of 150, 100, 50, 10, and 5 μg/mL. A sample (50 μL) was transferred manually into a vial on the appropriate rack of the autosampler. Each concentration was averaged over three measurements.

RESULTS AND DISCUSSION

Transducer Coating, Characterization, and Stability. To demonstrate the applicability of RIfS for monitoring biotechnological processes, the design of the sensor was based on the high binding specificity of glycopeptide antibiotics to D-Ala-D-Ala peptides,[10] which represent an excellent ligand−receptor model. Vancomycin was used as a reference for the characterization of the biosensor and the exemplary demonstration of the sensor principle. For the final calibration of the fermentation monitoring, we transferred the results to our ongoing research interests concerning the biosynthesis analogues of vancomycin, especially balhimycin.[18]

Common procedures for the preparation of suitable transducers are carried out in three steps. In the first step, the glass surface is silanized with GOPTS in order to obtain a reactive oxirane group for further modifications. In the second step, the silanized surface is coated with AMD and DAPEG (Figure 2), respectively, to suppress nonspecific adsorption of analytes or contaminants to the silica surface. To obtain reproducible results, the absence of nonspecific binding is crucial for all label-free detection methods. A spacer molecule (glutaric acid) connects the covalently N^{ϵ}-linked sensitive ligand peptide (N^{α}-acetylated L-Lys-D-Ala-D-Ala) with the polymer coating (Figure 2).

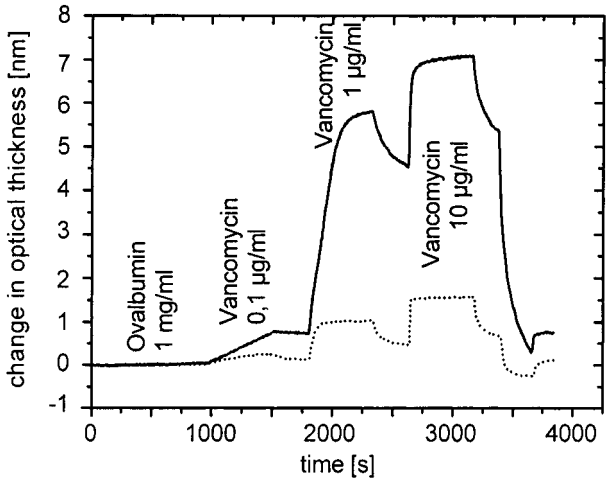

Figure 3. Characterization of Ac-Lys-$(D\text{-Ala})_2$-AMD (solid line)- and Ac-Lys-$(D\text{-Ala})_2$-DAPEG (dotted line)-modified transducers, respectively, with vancomycin. For both transducers types, three different concentrations of vancomycin were measured. For both AMD and DAPEG polymers, the nonspecific binding of the control (chicken ovalbumin) is negligible. The Ac-Lys-$(D\text{-Ala})_2$-AMD transducers show a higher binding capacity than the Ac-Lys-$(D\text{-Ala})_2$-DAPEG transducers because of the hydrogel structure of the dextran matrix. For the higher vancomycin concentrations (1 μg/mL, respectively, 10 μg/mL), equilibration is only reached for the Ac-Lys-$(D\text{-Ala})_2$-DAPEG-modified surface, indicating different kinetic and diffusional behavior for the binding of vancomycin to Ac-Lys-$(D\text{-Ala})_2$ immobilized on the two different surfaces.

Among the commonly used coating materials for the suppression of nonspecific binding we considered DAPEG and AMD as most suited, since upon injection of a blocking agent (chicken ovalbumin (1 mg/mL)), these polymers gave a response below 20 pm change in optical thickness. In contrast, typical signals of analyte binding were at least 10−100 times higher. Thus, nonspecific binding was considered to be negligibly small.

The determination of the response characteristics of the two DAPEG and AMD precoated Ac-Lys-$(D\text{-Ala})_2$-transducer types was performed by incubation with different concentrations of aqueous vancomycin solutions as the reference analyte. Intermediate transducer regeneration was performed with dilute hydrochloric acid (pH 1.5) (Figure 3).

Dependent on the polymer coating, Ac-Lys-$(D\text{-Ala})_2$-AMD or Ac-Lys-$(D\text{-Ala})_2$-DAPEG, we observed different response characteristics toward the analyte. For the Ac-Lys-$(D\text{-Ala})_2$-AMD transducer, a higher equilibration loading was achieved than for the Ac-Lys-$(D\text{-Ala})_2$-DAPEG-coated transducer. This behavior is based on the hydrogel character of the AMD matrix resulting in a flexible three-dimensional structure. Hence, AMD comprises additional binding capacities and allows the formation of more than one monolayer of the glycopeptide. In contrast, the Ac-Lys-$(D\text{-Ala})_2$-DAPEG coating is assumed to form a more rigid *polymer brush*, allowing the formation of a single monolayer of analyte on the transducer surface.

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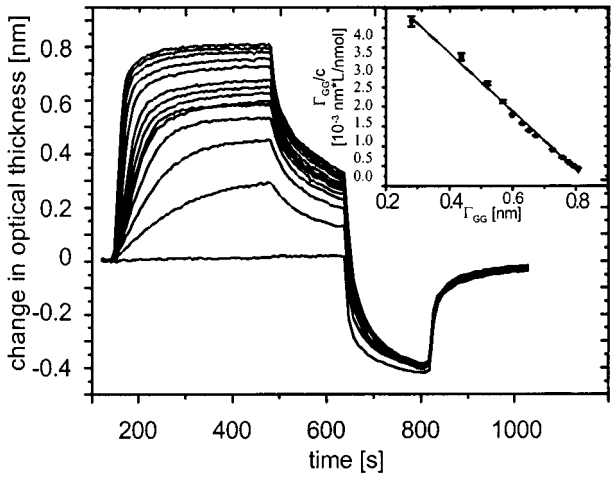

Figure 4. Determination of a calibration curve for the binding of vancomycin to Ac-Lys-(D-Ala)$_2$ linked to DAPEG transducers by Scatchard analysis. Error bars indicate the standard deviation of three measurements. Binding curves for different concentrations of vancomycin are shown. The inset shows the Scatchard analysis, yielding an affinity constant of $K = (7.42 \pm 0.18)$ M^{-1}. A determination of the on- and off-rates was not possible because of the influence of mass transport.

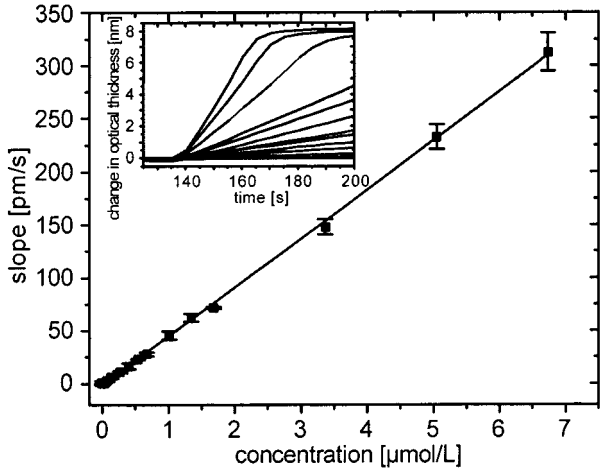

Figure 5. Determination of a calibration curve for the binding of vancomycin to Ac-Lys-(D-Ala)$_2$ on AMD transducers by evaluation of the initial slope of the binding curves. Error bars indicate the standard deviation of three measurements. Even for a surface coverage up to 70% of the saturation level, a linear relationship between the concentration and the initial slope of the binding curve was found.

The Ac-Lys-(D-Ala)$_2$-DAPEG-modified transducer reaches an equilibrum at higher glycopeptide concentrations in water (1 and 10 μg/mL) within 500 s. In contrast, for the Ac-Lys-(D-Ala)$_2$-AMD-modified surface, no equilibrium was observed, which indicates a different kinetic behavior for the binding of vancomycin to the Ac-Lys-(D-Ala)$_2$-AMD sensor. These differences are based on the chemical structure of the two polymers resulting in different diffusion parameters dependent on the used polymer matrixes. However, this may also indicate different accessibility to the recognition sites on the transducer surface. A comparison of the Ac-Lys-(D-Ala)$_2$-AMD- and Ac-Lys-(D-Ala)$_2$-DAPEG-coated transducer response using three different analyte concentrations is presented in Figure 3.

The second crucial point in biosensors is the reproducibility and the stability of the polymer coating. Both transducers showed a definite responding performance to standardized solutions of analyte and could be regenerated for more than 200 times (data not shown). According to these results, the transducers display sufficient stability for the monitoring of fermentation processes.

Analyte Quantification Studies with the RIfS Biosensor. Besides specific recognition of the analyte, reproducibility of the sensor, and stability of the coating, a reliable and reproducible quantification with sensor systems is mandatory. To evaluate their potential for quantification, calibration curves were recorded for both transducer types, which were, depending on the transducer coating, determined in two different ways.

Method A relies on the determination of the affinity constants for the binding of glycopeptide antibiotics to immobilized Ac-Lys-(D-Ala)$_2$-sensor peptide and is suitable for Ac-Lys-(D-Ala)$_2$-DAPEG-modified transducers. In this case, the steady-state coverage is according to the Langmuir isotherm, depending on the concentration of the glycopeptide, the saturation level, and the affinity constant K. The determination of the affinity constant was done by Scatchard analysis. Figure 4 shows the binding signals and Scatchard analysis for the analyte vancomycin, yielding an affinity constant of $K = (7.42 \pm 0.18)$ M^{-1}. This value is similar to those determined by Lahiri et al.[19] using surface plasmon resonance (SPR). The determination of the on /off rates could not be performed for reasons described by Lahiri et al.[19] In brief, all these measurements are strongly influenced by mass transport even for measurements performed with very low surface capacities for which mass transport usually is not the limiting factor. With a saturation level of $\Gamma_{max} = 0.85$ nm (Figure 4), a working range up to a vancomycin concentration of $\sim$0.67 μmol/L appears to be reliable. Arising from calculations for a transducer with the highest achievable surface capacity ($\Gamma_{max} = 1.5$ nm), this working range may be increased to a glycopeptide antibiotic concentration of $\sim$1.34 μmol/L. The limit of detection (LOD) was reached at 6.4 nmol/L (3σ criteria); the limit of determination (LOQ, limit of quantification) was found at 23.92 nmol/L (10σ criteria).

Method B is based on the evaluation of the initial slope of the binding curve and was preferred for quantification studies with Ac-Lys-(D-Ala)$_2$-AMD transducers displaying slow binding characteristics. Under mass transport limiting conditions (i.e., the binding to the surface is much faster than the mass transport by diffusion to the surface), the initial slope is proportional to the momentary concentration of the analyte in the flow cell, described by first Fick 's law. These conditions are usually present for low analyte concentrations and low surface coverage (<20% of saturation level).[20,21] Figure 5 shows the calibration for the analyte vancomycin. We observed a linear correlation between the concentration of the analyte and the initial slope of the binding curve, determined by a least-squares fit even for a surface coverage up to 70% of the saturation level. Hence, under the applied conditions, there is still mass transport limitation indicating a high association rate in accordance with the measurements on PEG-coated surfaces. For this calibration method, the LOD was at 51.9

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nmol/L (3σ criteria), the LOQ was at 173.0 nmol/L (10σ criteria), and the sensitivity was at 0.05 (pm/s)/(nmol/L). The upper concentration limit was at 6.7 μmol/L. For higher concentrations, the linear part of the binding curve consisted of less than five measure points and we resigned from determining the slope by linear regression.

Similar results were obtained for the analyte balhimycin, which is structurally similar to vancomycin. These results show that both transducer types are suited for the quantification of glycopeptide antibiotics. However, the Ac-Lys-(D-Ala)$_2$-AMD-coated transducer seems to be more suited for fermentation monitoring, since it displays particularly for low concentrations a higher signal-to-noise ratio and higher dynamic range. As a consequence, we used the Ac-Lys-(D-Ala)$_2$-AMD coating for all subsequent experiments concerning the characterization of fermentor broths.

Fermentation Monitoring. Besides all aspects of specificity and quantification shown in the results above, the decisive proof for the applicability as a biosensor is its exposure to fermentor broths containing highly complex compound mixtures, which may lead to unpredictable and unwanted interactions with the sensor. Hence, the goal to be reached includes a minimal number of preparation and purifications steps; preferably none has to be performed. At the current stage of our sensor development, samples containing balhimycin (produced by *A. mediterranei*) were taken at definite times and prepared for injection into the FIA system only by a short centrifugation step to obtain a particle-free sample.

The calibration was performed according to method B resulting in a LOD of 13.5 nmol/L and a LOQ of 47.1 nmol/L (data not shown). Since the concentrations of balhimycin raised to 200 μmol/ml at the end of the fermentation, all samples were diluted 100-fold to stay within the dynamic range of the transducer (Figure 5).

Although the characterization of the transducer with chicken ovalbumin as a control showed only negligible nonspecific binding, the injection of crude fermentor samples taken at the beginning of the fermentation process ($t = 0$) resulted in a binding curve (Figure 6) strongly influenced by nonspecific binding, by the change of the bulk refractive index after injection of fermentor samples, or by both.

It turned out that dilution of the samples as described above was crucial not only to stay within the dynamic range of the transducer but also to reduce unspecific binding to a nonobservable level. The minimal detection level of glycopeptide in fermentation media for diluted samples was determined for concentrations below 1.35 μmol/L. However, the *effective* LOD and *effective* LOQ, respectively, are sufficient for an excellent fermentation monitoring.

Since the RIfS biosensor detects all components within the fermentor that bind specifically to D-Ala-D-Ala, there is no discrimination between those substances. In consequence, even precursors of the glycopeptides that may feature the tricyclic structure are determined.

To reference the results obtained by RIfS, all samples were independently analyzed by HPLC−ES-MS analytics. In fermentor samples growing under the above-mentioned media conditions, three glycopeptide antibiotic metabolites (balhimycin, balhimycin V, deglucobalhimycin) with balhimycin as the major compound

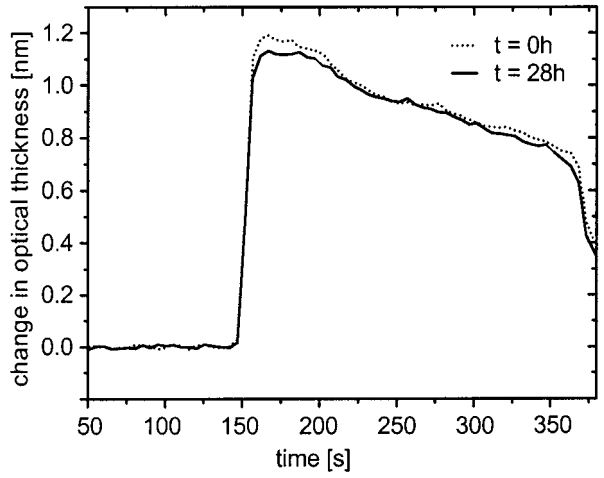

Figure 6. Injection of samples of crude fermentation broth, taken directly (0 h) and 28 h after inoculation of the fermentor (before vancomycin production started). A high signal indicating nonspecific binding to the surface, r changes in the bulk refractive index, or both is shown although the test for nonspecific binding with chicken ovalbumin (Figure 3) showed no nonspecific interactions with the surface.

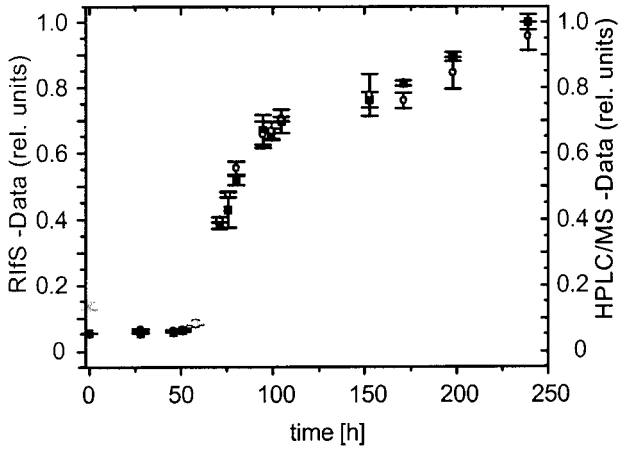

Figure 7. Monitoring of the balhimycin production during a fermentation process detected with both RIfS (black) and HPLC−ESI-MS (gray). Error bars indicate the standard deviation of three measurements. All samples were taken manually, centrifuged, and diluted by a factor of 100.

were detected with mass spectrometry. Since it was found that different glycosylation of glycopeptide antibiotics had no major effect on the ionization yields, these were considered to be equal. Based on this approximation, quantification of the fermentor samples was performed with ESI-MS. The corresponding results of both methods, HPLC−MS quantification and the RIFS sensor, prove the reliability of the presented approach. Figure 7 shows the balhimycin production during the fermentation process measured with both RIfS and HPLC−ES-MS detection.

CONCLUSIONS

We reported the design and development of a biosensor based on the optical label-free detection method RIfS for the detection and the quantification of analytes in biotechnological processes. Exemplified at the ligand−receptor model of glycopeptide antibiotics and their specific D-Ala-D-Ala ligand moiety, we showed that RIfS is a rapid and highly sensitive method for this application.

Besides the excellent reproducibility and the stability the method excels by low costs of the required equipment and in environmental terms without need or waste of chemicals. Nonspecific interactions were suppressed by using aminodextran- or diaminopoly(ethylene glycol)-coated transducers. Because of a more pronounced signal-to-noise ratio, the Ac-Lys-(D-Ala)$_2$-AMD-modified transducers were more suited for the fermentation monitoring. In addition, the AMD-modified transducer displayed a superior response characteristic. With the AMD-modified transducer, concentrations were determined within a few seconds by initial slope evaluation while for the DAPEG-modified transducer the equilibration state has to be reached. Results obtained with RIfS in quantification studies of fermentor samples were independently confirmed by HPLC−ES-MS.

As the only purification step, fermentor samples were centrifuged to obtain a particle-free solution. To build up a real on-line monitoring setup in fermentors, current studies are focused on a bypass system with filter unit and dilutor. Furthermore, we are working on the transference of this concept to other processes with specific ligand−receptor interactions.

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