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Disposable Optical Sensor Chip for Medical Diagnostics: New Ways in Bioanalysis

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An optical sensor system is described which is particularly well suited for medical point-of-care diagnostics. The system allows for all kinds of immunochemical assay formats and consists of a disposable sensor chip and an optical readout device. The chip is built up from a ground and cover plate with in- and outlet and, between, of an adhesive film with a capillary aperture of 50 μm. The ground plate serves as a solid phase for the immobilization of biocomponents. In the readout device, an evanescent field is generated at the surface of the ground plate by total internal reflection of a laser beam. This field is used for the excitation of fluorophor markers. The generated fluorescence light is detected by a simple optical setup using a photomultiplier tube. Because of the evanescent field excitation, washing or separation steps can be avoided. With this system the pregnancy hormone chorionic gonadotropin (hCG) could be determined in human serum with a detection limit of 1 ng/mL. Recovery values were 86, 106, and 102% for 5, 50, and 100 ng/mL hCG, respectively. The SD in repeated measurements ($n = 10$) was 5.6%. Furthermore, the feasibility of the system in competitive-type immunoassays was demonstrated for serum theophylline. A linear calibration curve of signal vs theophylline between 1 and 50 mg/L was obtained. Recovery values varied between 118% (10 mg/L) and 81.0% (20 mg/L).

Today, immunoassay is the predominant analytical technique for quantitative determination of analytes in the field of laboratory medicine. The specificity of the analysis is provided by the antibody molecule which recognizes the corresponding antigen. One of the key features of most immunoassays is the solid phase which facilitates washing and separation steps and serves as a matrix for the immobilization of either the antibody or the antigen. Another important feature is the label used in the immunoassay. Most commonly, radioisotopes or, for example, fluorescent and enzyme labels are applied. In particular, the advent and use of enzyme labels paved the way for rapid immunoassay testing.

In comparison with complex and sophisticated immunoassay equipment used in laboratory medicine, rapid immunoassays are mainly manual, single-use devices that are particularly well suited for point-of-care (POC) testing. Thus, these assays can readily be applied when the measurement has to be performed in emergency rooms, in ambulances, at the bedside, or at the doctor's office. According to Hesterberg and Crosby,[1] rapid immunoassays are defined as those that produce a result in less than 30 min, that are rated as moderately complex or waived by the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88), and that can be completed without the aid of additional equipment. The four primary rapid immunoassay formats in use today are latex agglutination, horizontal flow devices, tangential flow devices (the so-called dipsticks), and optical immunoassays.[1] Up to now, one of the drawbacks of these test kits was the complicated handling procedure and often the lack of sensitivity. Moreover, in most cases, these tests are qualitative or semiquantitative although strong efforts are undertaken to eliminate these limitations.[2−4] An analytical device based on reflectometry, for example, was developed by Boehringer Mannheim (the "Cardiac Reader")[5] for the readout of troponin T and myoglobin rapid tests.

Some of the disadvantages of conventional rapid tests can be circumvent with the relatively new technology of the so-called biosensors. These devices consist per definition of a biological component that provides the required specificity and the transducer that generates the signal. Both components have to be in close contact with each other. The most successful biosensor to date is obviously the one for blood glucose determination.[6] Biosensors that take advantage of the antibody/antigen interaction are called immunosensors.[7] Similar to conventional immunoassays, these devices are based on an immobilized ligand and a labeled component. Biosensors are useful analytical devices in various fields such as process control (for a review see ref 8), environmental analysis,[9] and laboratory medicine (for a review see ref

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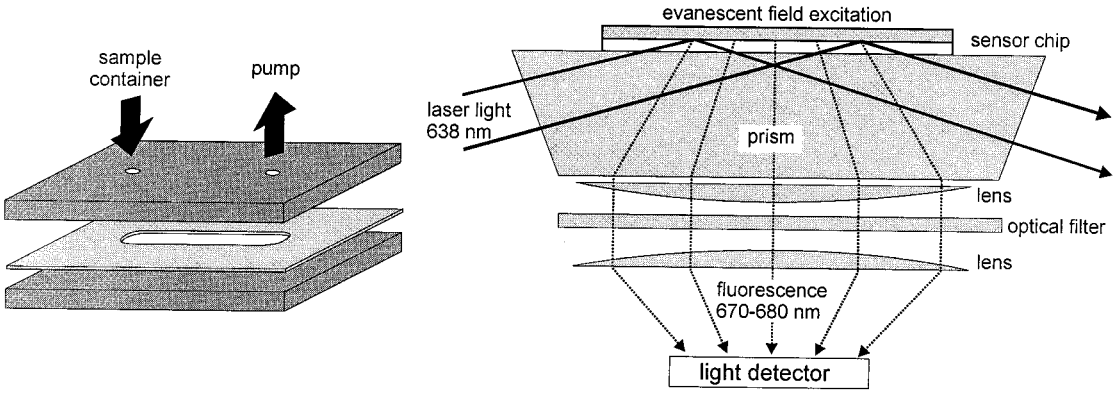

Figure 1. Setup of the optical sensor system. Left: scheme of the disposable sensor chip; right: setup of the readout device.

10). Another relatively new field of applications comprises arrays of nucleic acid hybridization sites, known as genosensors.[11] Here, applications range from gene-expression analysis to scanning for certain mutations.

Among the different types of transducer used for biosensors especially those which exploit changes in optical properties are perhaps the most promising ones. They detect, for example, changes in luminescence, fluorescence, refractive index, or protein coverage density. In the optical device described here, the proximity of biological recognition and optical detection is given, and it may thus be referred to as an optical immunosensor although it can also be referred to as a quantitative immunoassay system. The system consists of a disposable sensor chip and an optical readout device and combines the advantages of simple single-use test strips, e.g., simple handling, with the advantages of microtiter plate assays or automated laboratory equipment, i.e., high reproducibility, high sensitivity, and quantitative measurements. Whereas optical evanescent field fluorescence excitation avoids washing steps, the use of fluorescence markers in the long-wavelength range prevents interferences from the sample matrix. In addition, the design of the flow channel within the sensor chip allows for almost nondiffusion-limited mass transport and thus reduces assay times significantly.

Here, we describe the sensor system and its application for the determination of the pregnancy hormone chorionic gonadotropin (hCG) in human serum. Additionally, the feasibility of the system in competitive-type immunoassays is demonstrated for the drug theophylline. An application of the sensor system in the field of environmental analysis is described elsewhere.[12]

MATERIALS AND METHODS

Materials and Reagents. All fine chemicals were purchased from Sigma (St. Louis, MO) if not stated otherwise. The Cy5-Ab-labeling kit was purchased from Amersham (FluoroLinkTM-Cy5, monofunctional Dye, Braunschweig, Germany). Monoclonal antihuman chorionic gonadotropin (hCG) antibodies were purchased from Dunn Labortechnik (Asbach, Germany), antitheophylline antibodies (clone 3D9/5) were from Biomex (Mannheim, Germany), neutravidin was from Pierce (Rockford, IL). Theophyllin-8-butyriclactam was from Calbiochem (Bad Soden, Germany), whereas theophylline and hCG were obtained from Sigma (Deisenhofen, Germany). Precinorm U was obtained from Boehringer Mannheim (Mannheim, Germany) and used as standardized human serum.

Sensor Chip and Readout Device. The sensor systems consists of a disposable sensor chip and an optical readout device. A schematic setup of the sensor chip is depicted in Figure 1, left. The chip consists of a ground plate (0.25 × 10 × 30 mm poly(methyl methacrylate), PMMA), a cover with in- and outlet (3 × 10 × 30 mm PMMA), and between, an adhesive film with a capillary aperture of 50 μm. The ground plate serves as the solid phase for the immobilization of biocomponents, e.g., the capture antibody. At the inlet a sample container is mounted consisting of a 1-mL polyethylene (PE) pipet tip from Eppendorf Co. The outlet is connected to a syringe pump by a flexible silicon tube having an inner diameter of 0.8 mm and a length of approximately 200 mm. The sample is driven through the capillary gap by a flow rate of 1 μL/s. The sensor chip is optically connected to a prism of the optical readout device (Figure 1, right) by a refractive-index-matching fluid (Leica, Wetzlar, Germany. Oil for microscopy, $n_e^{23} = 1.518$). From one side a laser beam enters the prism and is reflected at the interface between the ground plate and the capillary aperture. Thereby the evanescent field is generated that is used for fluorescence excitation. The fluorescent light is collected by a lens glued onto the base of the prism forming a parallel light beam. This beam passes an optical interference filter to suppress the excitation laser light before the light is focused by a second lens onto the sensitive surface of a photomultiplier tube (PMT, Hamamatsu, M5773-01, Hersching, Germany). The photosensitive surface generates a current which is amplified approximately 1000 times inside the multiplier tube. At the output of the PMT the current is converted into a voltage signal and again amplified approximately 10^8 times. Consequently, the voltage signal increase in time is directly proportional to the amount of fluorophor bound to the surface of the flow channel. To avoid photobleaching of the fluorophor the power of the laser was adjusted to 0.06 W/mm^2. Under these experimental conditions, even after 10 min, no photobleaching was observed.

The measurement process is divided into two steps: (1) preincubation of sample and detector antibody and (2) measurement. In a sandwich assay, for example, the sample is mixed with

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a labeled detector antibody, thus forming analyte−antibody complexes. The fluorophor used in this study is Cy5, commercially available from Amersham, whose absorption and emission bands are in the red range of the light spectrum. Cy5 is excited efficiently by a low-cost, compact laser diode with an emission wavelength of 638 nm (Laser Graphics, LG 635-10L, Dieburg, Germany). The fluorescent light is in the range of 660−690 nm and only overlaid to a very small degree by the inherent fluorescence of the samples. After the preincubation period, the sample is pumped through the capillary aperture over the ground plate of the sensor chip.

By passing through this narrow flow channel, the analyte−antibody complexes bind to the capture antibodies immobilized on the surface of the ground plate, hence forming the so-called sandwich. The small capillary aperture ensures that the binding of the complex consisting of detector antibody and analyte is only insignificantly limited by diffusion processes. With an aperture of 50 μm and a flow rate of 1 μL/s the diffusion-limited layer is reduced to $\ll 1$ μm. Thus, the reaction kinetics of the binding process are predominantly dependent on the kinetic parameters of the affinity interaction but not on diffusion. For the excitation of the fluorophors the evanescent field of the laser light is used which is generated by total internal reflection of the laser beam at an interface between the high-refractive (n_1, PMMA, poly(methyl methacrylate)) and the low-refractive (n_2, liquid sample) material. The intensity of this field decreases exponentially with increasing distance from the interface in the half-space of the low-refractive (n_2) medium. The depth of penetration of the evanescent field depends on the angle of reflection and the refraction indices n_1 and n_2 of the materials used and has a typical value of approximately 150 nm. Consequently, only the fluorophor−antibody conjugates that have formed a sandwich together with the analyte and the capture antibodies are excited to fluorescence. Detector antibodies outside the evanescent field are not excited and consequently give no fluorescent light.

The sandwich complexes formed per unit of time are, in turn, only dependent on the detector antibody−analyte complex concentration and the binding rate, k_{on}, between this complex and the capture antibody. For one type of antibodies k_{on} is constant, and the sandwiches formed per unit of time therefore generate a time-related increase in the fluorescence signal which is directly proportional to the analyte concentration. Theoretical prediction and experimental results show that this increase is linear in the case of small analyte concentration and/or short times which always holds for the sensor setup presented here. Consequently, the slope of the linear increase is direct proportional to the concentration of the analyte. This method of measurement is highly reproducible and, in combination with long-range fluorophors, not susceptible to disturbances. Additionally, washing or separation steps can be avoided. From the increase of the fluorescence signal the initial slope (sensitivity), as V/s, is calculated. In general, every kind of immunochemical assay can be performed with this setup.

If not stated otherwise, lower limits of detection were calculated from the blank value (analyte concentration zero) plus 3-fold SD and interpolation on the calibration curve.

Immunoassays. Sandwich Assay for hCG Determination. The preferred assay format for hCG comprises neutravidin-coated sensor chips, a biotinylated monoclonal capture antibody, and a Cy5-labeled monoclonal detector antibody. In the assay, the analyte is preincubated with both antibodies. The reaction mixture with the sandwich complex is then pumped through the sensor chip. Here, the biotin label of the antibody facilitates the binding to the sensor surface. Because of the assay format a generic neutravidin chip could be used throughout all experiments. Spiked standardized serum and urine from male volunteers were used as samples throughout all experiments.

Synthesis of Antibody-Cy5 Conjugates. Cy5 labeling of the monoclonal anti-hCG antibodies was performed according to the manufacturer's instructions. One milligram of antibody (1 mg/mL in PBS, pH 9.3) was added to the preactivated Cy5-NHS and incubated for 2 h at room temperature. Unbound fluorophor was removed using FPLC chromatography with Sephadex G50. Using MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry), conjugation ratios (fluorophor/protein, F/P) were determined.

Biotinylation of the Capture Antibodies. For biotinylation, 2.2 mg of D-biotinyl-ϵ-amidocaronic acid-*N*-hydroxysuccinimidester was dissolved in DMSO. Subsequently, 50 μl of DMSO and 10 μl of the biotinylating reagent were added to 1.0 mg of the anti-hCG antibody in 120 μl of PBS buffer pH 7.4. After incubation for 2 h at room temperature, the excess of biotin was removed via a PD-10 column. Fractions containing the antibody were dialyzed against PBS buffer pH 7.4 overnight. The final protein concentration was 0.4 mg/mL for the anti-hCG antibody.

Competitive Assay for Theophylline Determination. The assay format for theophylline determination comprised monoclonal antitheophylline antibodies coated on the sensor chips and a theophylline−Cy5 conjugate. The human serum sample containing theophylline was mixed with the Cy5 conjugate. When applied to the sensor chip, theophylline in the sample competed with the labeled drug for binding to the immobilized antibody.

Synthesis of the Theophylline−Cy5 Conjugate. One milliliter of diaminoethane was added to 50 mg of theophylline-8-butyriclactam and incubated for 1 h at room temperature and overnight at 4 °C. Excess of diaminoethane was removed under vacuum. The residue was dissolved in methanol and dried under vacuum again. Thirty milligrams of a light yellow powder was obtained. Finally, 3.0 mg of the Cy5-NHS in 60 μl DMF were added to 10.3 mg of theophylline-8-butyricacid-aminoethyleamide in 300 μl DMF. The reaction was allowed to proceed for 1 day at room temperature.

Immobilization of Biocomponents on the Sensor Chip. According to the assay formats described above in the hCG assay neutravidin had to be immobilized on the surface of the sensor chip whereas a monoclonal antitheophylline antibody had to be immobilized for the theophylline assay. For immobilization of neutravidin 10 μL of a 1.0 mg/mL solution of the protein in PBS buffer pH 7.4 was incubated for 3 h at room temperature in the flow channel of the sensor chip. For the theophylline assay, 10 μL of a 0.1 mg/mL solution of the antitheophylline antibody in PBS buffer pH 7.4 was incubated for 24 h at 4 °C. After washing with PBS buffer, pH 7.4, the channel was dried in a nitrogen airflow. Prepared sensor chips were stored dry at 4 °C.

General Assay Protocol. Each measurement was performed with a different, single-use sensor chip. In the hCG assay a 100 μL sample (standardized serum) was mixed with a 100 μL cocktail containing 2.04 μg/mL anti-hCG−Cy5 conjugate and 0.91 μg/mL

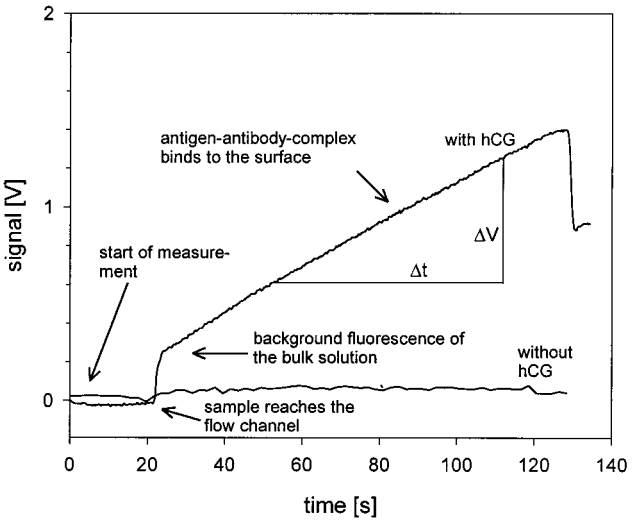

Figure 2. Typical sensor signal in the presence and absence of hCG. 100 μL sample at a flow rate of 1 μL/s. Initial signal intensities have been normalized to 0 V.

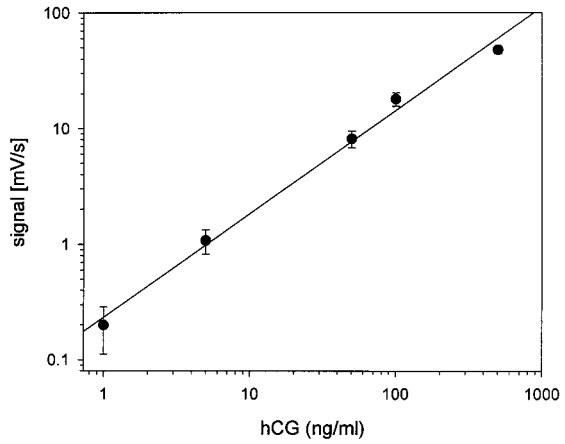

Figure 3. Calibration curve for human chorionic gonadotropin in serum. Mean values and SD of 3-fold determinations, $r = 0.993$.

anti-hCG−biotin conjugate (in PBS buffer pH 7.4, 1% BSA). The mixture was allowed to incubate for 5 min prior to the measurement.

In the theophylline assay a preincubation step was not required. The serum sample was diluted 100-fold with PBS buffer pH 7.4, 1% BSA. One hundred microliters of the diluted sample was mixed with 100 μL of the cocktail containing the diluted theophylline−Cy5 conjugate (1/15.000 in PBS buffer pH 7.4, 1% BSA). In the theophylline assay no preincubation time was necessary.

After mounting the sensor chip in the optical readout device and equilibration of the baseline (5−10 min) the mixtures were filled in the sample container of the sensor chip. The sample was then pumped with a flow rate of 1.0 μL/s through the sensor chip. The change of fluorescence intensity was recorded over the time with a sample rate of 2 Hz. After the measurement the disposable sensor chip was discarded.

RESULTS AND DISCUSSIONS

Sandwich Assay for hCG Determination. The fluorophor/protein (F/P) ratio of the Cy5-detector antibody conjugate was determined to be 3.4 and not further optimized. A typical sensor signal obtained for a sandwich immunoassay of hCG is shown in Figure 2. Here, a sample volume of 100 μl was used. At the start of the measurement at $t = 0$, the flow channel of the sensor chip is filled with air. It then takes approximately 25 s for the sample to move into the flow channel. A small step in the fluorescence signal due to background fluorescence of the bulk solution is observed after 25 s. Then the biotinylated capture antibody facilitates the binding of the immunocomplex to the neutravidin-covered surface, and an increase in fluorescence is observed from 25 to 125 s. If a sample without hCG is applied to the sensor (Figure 2), no such signal increase is observed. The increase in fluorescence is recorded for approximately 100 s, and a signal as V/s is calculated. After 125 s the signal decreases because the sample has been pumped completely through the chip, and the flow channel is filled with air again. The slope of the signal is only dependent on the kinetic constant k_{on} of the ligand−receptor system, certain optical parameters of the readout device, and the concentration of the analyte. As the former parameters are constant for one particular assay format, the slope of the signal is converted into a corresponding analyte concentration, once a calibration curve has been obtained. This procedure for signal generation has several advantages over the measurement of the fluorescence intensity at two certain times. Favorably, the analysis is independent of the absolute signal of the baseline and the step due to background fluorescence of the bulk solution.

In the hCG assay, we took advantage of the neutravidin−biotin system, as the monoclonal antibody used as capture could only partly be used for adsorptive immobilization (data not shown). The time required for the preincubation of analyte and antibodies was found to not be crucial. After a 1−2 min incubation no further signal increase was observed. Thus, the preincubation time was set arbitrarily to 5 min.

In the first experiments the influence of the sample matrix on the sensor signal was investigated. As expected, the specific signal in the presence of the analyte varies between different matrixes. Buffer, urine, and human serum samples spiked with 0.1 μg/mL of hCG showed signal intensities of 3.8, 3.0, and 2.5 mV/s, respectively. Consequently, calibration has to be carried out in the matrix in which the measurements will be performed. More important is the lack of background signals in the absence of the analyte. Neither serum nor urine affects the sensor signal. This beneficial feature of the system can be attributed to the data analysis via the slope of the fluorescence increase. Disturbances from the matrix can thus easily be eliminated.

A calibration curve for hCG in human serum is shown in Figure 3. With an incubation time of 5 min and ~2 min for the measurement, the whole assay takes 7 min only. The calibration curve covers more than 3 orders of magnitude. With a detection limit of 1 ng/mL the curve can thus be applied for hCG determination from the first week of pregnancy on.[13] The background signal in the absence of hCG was determined to be 2.88×10^{-2} mV/s.

The lower limit of detection expressed as international units per liter is 14 IU/L and thus only slightly above the hCG concentrations of healthy nonpregnant women (3−10 IU/L[14]). The

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concentration of hCG in serum starts to increase 7−11 days after ovulation. Thus, although not optimized in every respect, the sensor allows for hCG determination from the first week of pregnancy on and can compete with modern hCG tests. In general, these tests can measure elevated hCG concentrations of 20−25 IU/L and greater.[15] Because of the large linear range, even very high concentrations of approximately 1000 ng/mL (14.000 IU/L), as observed toward the end of the first and in the course of the second trimester, can be determined without additional sample dilution. Only the quantitative measurement of peak concentrations of 100.000 IU/L during weeks 8−10 would require a dilution step.

The detection limit of 1 ng/mL, i.e., 2.6×10^{-11} mol/L, is comparable to a number of reported heterogeneous hCG sandwich assays;[16,17] however, it has to be taken into account that the whole measurement takes a few minutes only and that no washing or separation steps are required. Immunosensors for hCG determination have been described as well.[18,19] An optical sensor which works with a planar waveguide system, for example, has been described as a fluorescence capillary fill device (FCFD[20]). For hCG, sensitivities of 4.5×10^{-11} mol/L have been reported. An amperometric immunosensor for hCG was developed by Meyerhoff et al.[21,22] The detection limit of 0.5 ng/mL of hCG is comparable to the results obtained here. A sensor system based on capacitance measurements of antibody−antigen interactions was reported by Berggren et al.[23]

Although each measurement was performed with a single-use sensor chip, the reproducibility is excellent. The results are even more impressive as each single chip was coated individually and as each chip was mounted by hand in the experimental setup. Thus, the errors in repeated measurements reflect not only variations in one assay (preparation of standards, dilutions, etc.) but are an expression of all possible sources of error. It is most likely that due to further automation, which is part of ongoing work, the reproducibility will be further improved.

The recovery of hCG from supplemented human serum samples was determined by using the calibration curve shown in Figure 3. Serum samples with 5, 50, and 100 ng/mL hCG were found to have 4.3 ± 0.6, 53.3 ± 7.2, and 102.0 ± 5.4 ng/mL hCG, respectively (each $n = 5$). This corresponds to recovery rates of 86 (5 ng/mL), 106.6 (50 ng/mL), and 102.0% (100 ng/mL). The standard deviation in repeated measurements of one serum sample was calculated to be 5.6% ($n = 10$, 50 ng/mL hCG).

Additionally, hCG was determined in spiked urine samples from male volunteers. As can be seen in Figure 4 an excellent correlation between the supplemented and the found hCG concentration in three different urine samples was observed. For the calculation of the concentrations from the sensor signals, a calibration curve obtained in urine was applied.

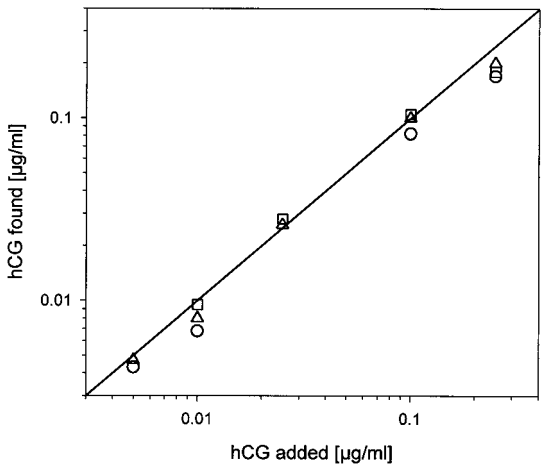

Figure 4. Recovery of hCG from supplemented male urine samples. The 3 different symbols represent 3 different urine samples.

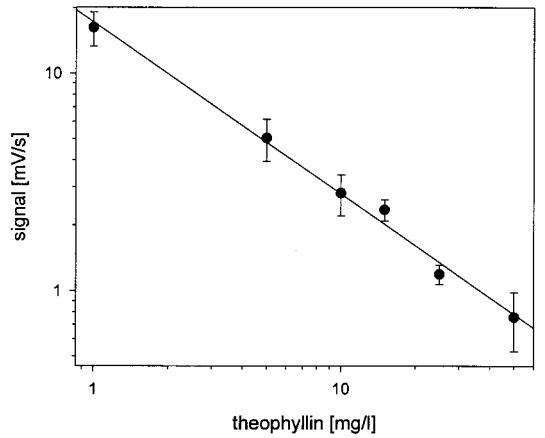

Figure 5. Calibration curve for theophylline in human serum. Mean values and errors of double determinations, $r = 0.993$.

Competitive Assay for Theophylline Determination. Theophylline was chosen as analyte in order to demonstrate the feasibility of the sensor system not only for sandwich-type immunoassays but for competitive formats as well. In the first experiments, the theophylline−Cy5 conjugate was titrated. On the basis of these experiments the conjugate was diluted 1/15.000 prior to the addition of the sample. The calibration curve for theophylline (Figure 5) covers the important clinical range of 10−20 mg/L for serum samples.[24] Comparable results were obtained by Chiem and Harrison[25] using a microchip system with integrated immunoreactor and electrophoretic separation. Recovery values for 15 mg/L serum samples were 107 ± 8%. Here, the recovery of theophylline from supplemented serum samples was determined with the calibration curve shown in Figure 5. Serum samples containing 10, 15, and 20 mg/L theophylline were found to have 11.8, 13.9, and 16.2 mg/L, respectively. This corresponds to recovery rates of 118.2 (10 mg/L), 92.7 (15 mg/L), and 81.0% (20 mg/L).

In conclusion, the results have illustrated that the proposed sensor system is feasible for applications in the field of in vitro

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diagnostics, in general, and for the performance at the point-of-care in particular. Assays are easy to perform, with quantitative results being delivered usually within a few minutes. Moreover, the system offers additional features that are under on-going investigation. First, the chip, including the prism, as the disposable part will be manufactured by injection molding with very low manufacturing costs. From the automated production a further improvement in reproducibility can also be expected. Second, the reagents required for the assay will be contained within the chip. Here, membranes as used in immunochromatographic test strips will be integrated into the chip. These membranes will be used for the separation of cellular compounds from whole blood or for conjugate release. The system will thus be self-contained, and sample preparations will become obsolete. Finally, and this is one of the major advantages, different receptor molecules, e.g., different capture antibodies can be immobilized on different spots of the flow channel. A simple moving shutter between the chip and the optical detector facilitates than the simultaneous measurement of as many as 10 analytes with one sensor chip. A paper about the simultaneous quantitative determination of two cardiac markers in blood samples is in preparation. Thus, the system described complies with the general requirements and desirable features of POC testing devices and provides an ideal generic technology for all kinds of immunochemical assays.

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