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Immunosensor for the Measurement of Human Serum Albumin in Urine Based on the Spreeta Surface Plasmon Resonance Sensor

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The application of SPR for measurement of the concentration of human serum albumin (HSA) in urine was studied using the compact integrated SPR sensing system Spreeta. HSA was immobilized via cystamine and glutaraldehyde onto the gold sensing area and a competitive assay for HSA was developed using a limited amount of the monoclonal antibody AL-01 in solution. Measurements were carried out in the flow-through mode and the interaction between immobilized HSA and antibody was observed in real time. To obtain reproducible results, different conditions of the measurement (method of immobilization of HSA, data evaluation, concentration of antibody, regeneration procedure) were tested. The calibration curve for clinically relevant concentrations of HSA in urine samples was constructed using 300-times diluted antibody in the form of ascites fluid. The measuring range was between 0.1 and 5 mg/l of HSA, the sensing surface was successfully regenerated and suitable for more than 20 assays. The developed method was tested on real samples of urine; to overcome the non-specific adsorption of urine components, the differential approach was adopted and the measured signal was corrected by subtraction of the response observed in the absence of the antibody.

Keywords: Surface plasmon resonance; Human serum albumin; Immunosensor; Albuminuria

INTRODUCTION

Albuminuria often accompanies diabetes mellitus, the most common cause of end stage renal disease (ESRD), resulting in about one-third of new ESRD cases. The development of the late nephropathic complications can be prevented when the early stage of the disease is identified. Regular screening of urine for albumin excretion is recommended as part of the routine patient review in insulin-dependent diabetes mellitus targeted intervention. In this way, progression of nephropathy is prevented and the cardiovascular system of patients showing early indications of renal disease becomes protected [1].

The most widely used screening test for albuminuria in clinical laboratories is the standard "dipstick" test—the dye-binding method [2]. Sensitive strips detecting albuminuria as low as 20 mg/l are available for clinical use. Such strips provide a semi-quantitative measure of albumin excretion in the normal/low microalbuminuria range, however, the assay is not specific for albumin only. Immunoassay technology represented by ELISA [3] now permits specific detection of urinary albumin at concentrations below 1 mg/l. However, trained personnel and laboratory conditions are necessary for this method and the time of analysis is longer than 1 h.

Immunosensors (immunochemical biosensors) are based on the highly specific interaction between an antibody and the corresponding antigen. One of the binding molecules has to be immobilized on the active surface of a suitable physico-chemical sensor (transducer), whereas the partner to be detected is free in solution. Direct (label free) and indirect measuring formats are utilized; the former ones employ quite a simple measuring procedure, the latter usually exhibit significantly higher sensitivity [4]. The first attempts to construct immunosensors (bioelectrodes) for albumin started in the 1970s [5]. Up to now, several approaches have been described. The piezoelectric flow-through immunosensor [6]

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reported a measuring range from 0.1 to 100 mg/l and the time of analysis was above 30 min; however, real samples were not analyzed. The piezoelectric immunosensor employing latex agglutination [7] detected albumin levels above 100 mg/l. Immunoassay for albumin employing final voltammetric detection of metal chelate labels achieved a detection limit of 1.8 mg/l in serum, however, the complete procedure lasted for more than 2 h [8]. An interesting direct amperometric immunosensor used antibodies incorporated inside electropolymerized layers; quite a fast response to albumin (5 min) and a reversible response were achieved in flow mode using pulses of potential [9]. Another amperometric immunoelectrode used antibodies immobilized on plasma-treated membranes [10]. The disposable piece of membrane was attached to the H_2O_2-measuring electrode and albumin–glucose oxidase conjugate served as a tracer. The operating range was from 0.5 to 100 mg/l albumin in urine; an analysis time close to 6 min was achieved. Recently, piezoelectric and surface plasmon resonance-based systems were used to study the interaction of human serum albumin (HSA) with antibody but without any analytical results [11]. To the best of our knowledge, no immunosensor system for albumin has been developed as far as the final stage of a commercial device.

In the previous paper, we have described the development of the piezoelectric immunosensor for HSA [12]. Due to successful results for indirect measurement of HSA concentration in samples, the same method was applied here on the commercially available Spreeta optical sensor from Texas Instruments. Spreeta is a highly integrated, compact, and miniature device based on the surface plasmon resonance phenomenon. The economically affordable cost makes it suitable for widespread use in different situations including clinical analysis.

EXPERIMENTAL

Materials and Methods

Albumin (HSA), cystamine dihydrochloride and Tween-20 were from Sigma (St Louis, USA). The monoclonal (MAb) anti HSA antibody AL-01 (EXBIO Praha, Prague) [13] was obtained as crude ascites fluid (protein content 35.9 g/l, IgG content 2 g/l). Glutaraldehyde was from Reanal (Budapest, Hungary). Deionized water (Millipore apparatus) was used for all experiments.

Preparation of the Immunosensing Surface

The gold surface of SPR sensors was washed using detergent (Triton X-100, 10% in 0.1 M NaOH), incubated for 2 h at room temperature in cystamine (20 g/l in water) and then washed in distilled water. The amino groups from the self-assembled cystamine monolayer were activated with glutaraldehyde (3% in water, 1 h incubation at room temperature). The final step was incubation with HSA (500 mg/l in phosphate buffer, 0.05 mol/l, pH 7.0) overnight at 4°C. After washing and drying, the sensors were ready for measurements. To evaluate the individual modification steps, the flow-through mode was chosen and the SPR signal was recorded. On the other hand, routine immobilization procedures were carried out using a static incubation arrangement because of the longer contact of biocompounds with the surface.

Instrumentation

The electronic control unit, flow-through cell, exchangeable Spreeta sensors and data handling software were supplied by Texas Instruments (Dallas, USA). The SPR Spreeta sensor contains a near infrared light source that is polarized to enhance surface plasmon resonance, a gold sensing surface, a mirror and a linear array of 128 silicon photodiodes. The entire assembly is encased in an optically clear material. Except for the sensing surface, the sensor is coated with an opaque material to block out external light. The sensor fits to a miniature flow cell consisting of PTFE body with holes for inlet, outlet, a temperature sensor and a stainless steel mounting plate, the schematic view is shown in Fig. 1. The assembled system should be placed inside a closed black box for optical isolation. The input tube to the flow-through cell was connected to a Minipuls MP3 peristaltic pump from Gilson (Villiers Le Bel, France) providing a constant flow rate of 50 μl/min for all experiments. Silicone tubes (inner ∅ 0.16 mm) were used for all connections. The measurements were performed at room temperature (25 ± 1°C) using PBST (0.05 mol/l phosphate buffer pH 7.0,

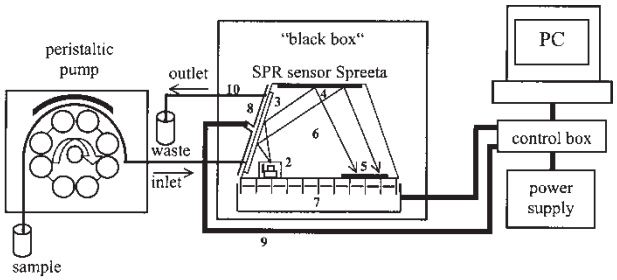

FIGURE 1 Scheme of experimental set-up: (1) LED and aperture, (2) polarizer, (3) gold sensing film, (4) gold mirror, (5) linear silicon photodiode array, (6) clear material, (7) cable connector and chip holder, (8) stainless steel mounting plate, (9) temperature sensor pins and (10) silicone tubes.

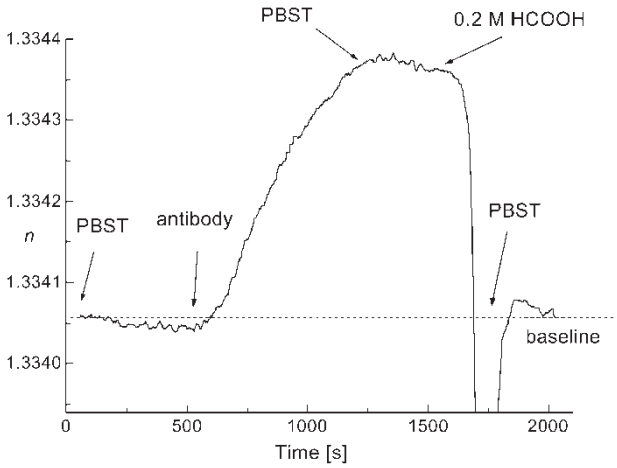

FIGURE 2 Scheme of the measuring procedure; the arrows denote addition of buffer, antibody and regeneration solution, respectively.

0.1 mol/l sodium chloride and 0.05% Tween-20) as the working solution.

Measuring Procedure

One measuring cycle consisted of the following steps (see also Fig. 2):

1 min flow of PBST to achieve a stable initial signal,
10 min flow of the sample (containing fixed addition of IgG) providing increase of signal (association phase),
5 min flow of PBST to initiate dissociation of the antibody-albumin immunocomplexes (dissociation phase),
1 min flow of 0.2 mol/l formic acid to regenerate the sensing surface.

The refractive index was the measured parameter; the initial calibration of the sensor was performed according to the recommendations of the manufacturer. All measurements were carried out indirectly using the chip with immobilized HSA employing the competitive immunoassay format; the sample consisted of a mixture of either albumin standard or urine (diluted 10–100 ×) and a constant concentration of anti HSA MAb (ascites fluid, 300-times diluted); preincubation for 5 min was carried out in order to allow formation of immunocomplexes in the mixture.

Kinetic Calculations

To better characterize the antibody AL-01, the equilibrium dissociation constant K_D was determined. The values of the refractive index n_{eq} were recorded at the end of each binding curve in the following buffer zone with stable signal. A hyperbolic function was fitted to the n_{eq} vs. c, the concentration of IgG, according to the equation:

$$n_{eq} = \frac{n_{max} c}{K_D + c} \tag{1}$$

where n_{max} is equal to the maximum change of the refractive index—binding capacity of the surface.

For the competitive assays, the dependence of the relative change of refractive index n on of the mass concentration of the analyte γ was fitted to the sigmoidal equation:

$$n = A_2 + \frac{A_1 - A_2}{1 + \exp[(\gamma - \gamma_0)/w]} \tag{2}$$

where A_1, A_2, γ_0 and w are parameters of the fitted equation, γ_0 indicates the middle point of the assay. The non-linear regression module from Origin (Microcal, Northampton, USA) was used for all numerical fitting calculations.

RESULTS AND DISCUSSION

Characterization of the Immunosensor

The control program of the Spreeta system provides different signal evaluation methods based on a specific part of the recorded SPR curve[†]: (1) 1st moment below a baseline, (2) point of specific reflectance, (3) polynomial fit about the minimum, (4) zero crossing of the first derivative. The *first moment* method calculates the first moment of the SPR curve below a baseline. Only parts of the SPR curve that are below the baseline are included in the calculation according to the equation:

$$\text{First moment} = \frac{\sum_{i=1}^{128} |\text{SPR\_}signal_i - \text{Baseline}| * i}{\sum_{i=1}^{128} |\text{SPR\_}signal_i - \text{Baseline}|} \tag{3}$$

where the summations exclude all data points where the SPR signal is greater than the baseline. The *point of specific reflectance/signal* method looks for a pixel position at which the SPR curve equals a specific value. The *polynomial fit about the minimum* method makes a first pass of the SPR curve to find the approximate location of the minimum of the resonance. Next, it performs an n-th order polynomial least squares fit to interpolate the position of the minimum. The *zero-crossing of the first derivative* method is based on change of sign of the first derivative close to the minimum of the SPR curve. All these methods were applied to the evaluation of SPR binding curves for 1000 × diluted monoclonal antibody against HSA. Each method provided a

[†]Operation Manual, Spreeta Experimenter's Kit; Texas Instruments, Inc., 1998, Dallas, TX, USA; http://www.ti.com/spreeta.

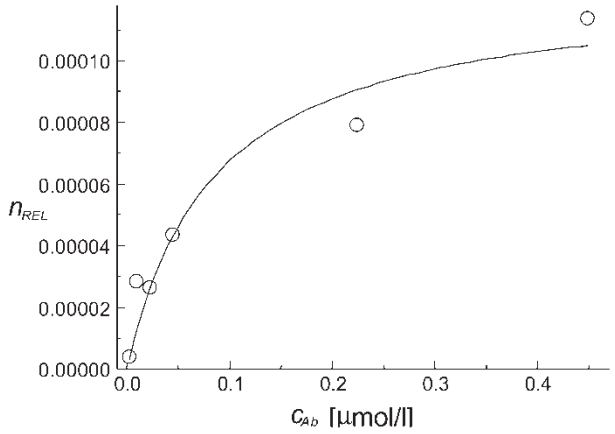

FIGURE 3 Dependence of the refractive index change (relative to the baseline) on the increasing concentration of the antibody. The solid line represents fit to the hyperbolic equation characterized by the equilibrium dissociation constant $K_D = 0.085 \pm 0.045\,\mu mol/l$ and the binding capacity of the surface represented by maximum change of the refractive index $n_{max} = 0.00012 \pm 0.000020$.

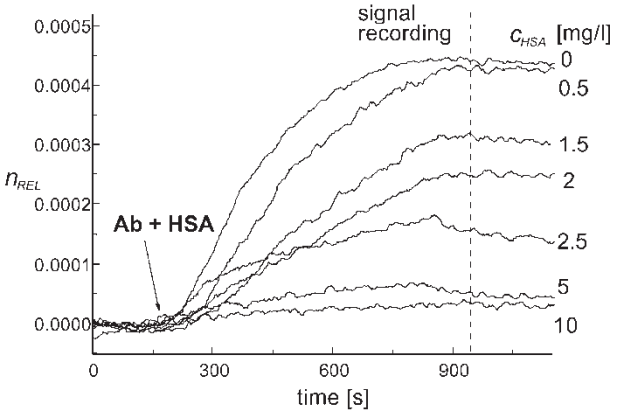

FIGURE 4 The example of binding curves for calibration mixtures with constant concentration of antibody (300-times diluted ascites fluid) and variable concentration of HSA (indicated close to the curves). The arrow indicates addition of the mixture; the vertical dotted line indicates position of signal reading for calibration.

different signal due to the reaction of antibody. The methods differed in sensitivity, noise and analysis rate. The best results were obtained using the method *1st moment below a baseline* and this approach was used for the following measurements. The other methods provided higher levels of noise and in selected measurements even failed to provide reasonable signal.

The chosen immunochemical procedure was based on the previously published piezoelectric biosensor [12]; the only difference was in concentration of the regeneration reagent for restoration of the biosensitive layer. The concentration for the formic acid was lowered to 0.2 mol/l and the complete dissociation of immunocomplexes at the sensing surface was achieved within 60 s. One SPR biosensor was used for 40 measurements. The possibility of total removal of all biolayers from the gold film was studied. The 1-h regeneration using chromic acid followed by a 1-h regeneration using 10 mol/l sodium hydroxide resulted in a fresh gold surface. The biosensor based on the regenerated gold surface exhibited an approximately three-fold increase of the binding capacity for antibody; unfortunately, the competitive reaction was significantly limited and albumin in solution was not able to prevent binding of the antibody to the surface. Therefore, the total regeneration of the SPR chips was not further considered.

To characterize the affinity of the antibody, the binding of increasing concentrations of antibody on the surface modified with HSA was followed similarly as in Fig. 2. The equilibrium responses obtained in this way are plotted against the corresponding molar concentration of antibody in Fig. 3. The obtained dependence was fitted to Eq. (1) and the obtained parameters were used to calculate the kinetic equilibrium association constant $K_A = K_D^{-1} = 1.18 \times 10^7\,l\,mol^{-1}$.

Competitive Measurement of Albumin

To measure the concentration of HSA by using the competitive assay, the calibration of the immunosensor was carried out using standards of albumin in buffer. A variable amount of HSA was pre-incubated with a constant concentration of MAb (ascites fluid, concentration of protein 120 mg/l). The effect of preincubation time has been previously studied, and it was found that equilibrium between free HSA and free MAb was established within 10 min [12]. In order to shorten the total time of analysis, preincubation was carried out for 5 min only. The mixture of HSA standard with antibody was injected into the flow cell and the binding reaction followed for 10 min (Fig. 4). The maximum change of refractive index for antibody in the absence of HSA was equal to 3.34×10^{-4}. In the absence of antibody, a negligible response was observed. As signal, the change of the refractive index after a fixed time interval was used and the calibration curves for two different immunosensors were constructed (Fig. 5). For practical evaluation, the curves were approximated with the sigmoidal function [Eq. (2)]. The parameters obtained by this fitting are summarized in Table I. The mid-point γ_0 of the immunoassay was equal to 2.12 mg/l. The method was sensitive for concentrations between 1 and 10 mg/l. As evident from Fig. 5, quite a narrow working range was obtained thus limiting the working range of the assay. Two calibration curves were recorded for two different sensor chips, which provided slightly

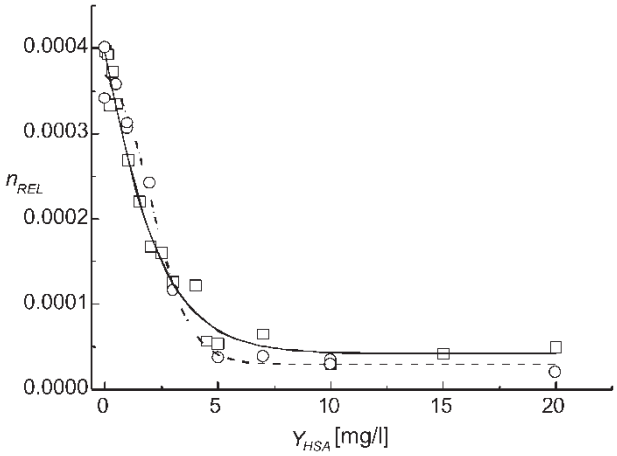

FIGURE 5 Calibration curves for the competitive immunoassay of HSA. The results obtained for two different immunosensors are shown: sensors no. 1 (squares) and no. 2 (circles).

different parameters. The manual handling of sensors during modification with biocompounds and different conditions during measurements are probably responsible for this result. It is important to note that the characteristics of the SPR sensor chips exhibited some minor fluctuations between individual units too.

Determination of Albumin in Urine Samples

The developed method was finally evaluated on the real samples of urine. The following procedure was carried out in order to find a suitable dilution for samples. Urine samples (from persons without health problems) were diluted in PBST 10×, 20× and 30× before injection to the flow cell with the SPR chip. Initially, samples involving only diluted urine samples without antibody were tested and the non-specific interaction of urine components with the sensing surface was observed and quantified. A steep increase of signal was observed after injection to the flow cell. After injection of buffer, a gradual decrease of the signal was observed and the non-specifically bound components were slowly completely released from the sensing surface.

When introducing urine samples pre-incubated with antibody, the non-specific adsorption increased the useful specific signal due to the binding of antibody. The possibility of the elimination of low-molecular components from urine using dialysis was previously studied [12], unfortunately, without any positive effect. For practical evaluation of the albumin concentration in samples, the subtraction of responses of the sample in the presence and absence of antibody was chosen as a simple method for correction of the non-specific interactions. For the following experiments, the real samples of urine from healthy persons were always diluted 10-fold. The results of evaluation are summarized in Table II. Different concentrations of HSA were determined for each sample; most results were below the threshold value for albumin in urine of 20 mg/l, the exception was sample no. 5 providing content above the threshold value.

TABLE I Parameters of the sigmoidal functions (Eq. (2)) approximating the calibration curves (relative response vs. concentration of albumin) for two individual Spreeta immunosensors for the competitive assay of albumin

Sensor	A_1	A_2	γ_0 (mg/l)	w (mg/l)
Sensor no. 1	0.00116	0.0000423	1.13	1.71
Sensor no. 2	0.000395	0.0000296	2.12	0.840

TABLE II Concentration of albumin in urine samples from healthy individuals determined using the SPR Spreeta sensor

Sample no.	1	2	3	4	5
pH	6.0	5.5	5.5	5.0	5.5
Albumin (mg/l)	0.270	18.9	0	1.68	34.7

For validation of the immunosensor, another set of real samples with a known concentration of protein was used. The levels of protein were determined using the standard Roche Diagnostics Urinary/CSF protein assay (Hitachi analyzer) in the official clinical laboratory of the Hospital in Vyškov. The assay is based on the method described by Iwata and Nishikaze [14], later modified by Luxton [15]. In this method, benzethonium chloride reacts with protein in a basic medium to produce a turbidity that is more stable and evenly distributed. The direct assay shows similar reactivity to albumin and γ-globulin, and no interference due to short peptides. Interference from magnesium ions is eliminated by the addition of EDTA. U/CSF Protein determinations can be performed as either the endpoint assay with sample blank or as kinetic assay. The measuring range for the endpoint assay is 20–2000 mg/l and for rate assay is 60–2000 mg/l of protein.

Five different samples of the patients from the hospital were analyzed using both standard protein assay and using the Spreeta immunosensor. For the immunosensor analysis, it was necessary to dilute samples 100× in order to fit within the calibration curve. The obtained results are summarized in Table III (Fig. 6). The concentration of albumin obtained using the SPR immunosensor correlated with the protein contents determined using the standard analytical method. However, for the samples with very high protein concentrations (no. 8 and 9), the albumin levels were lower than expected; some other proteins were probably present

TABLE III Analysis of real urine samples of five different patients form the hospital: comparison of the standard clinical assay (protein) and the Spreeta immunosensor (albumin). The immunosensor was also used to analyze samples after spiking with albumin (200 mg/l added)

Sample no.	6	7	8	9	10
pH	7.0	5.5	5.5	5.0	8.5
Hitachi-Protein (mg/l)	210	470	1160	1000	460
Spreeta-Albumin (mg/l)	157	202	445	322	306
Spreeta-Albumin (mg/l) (with addition of HSA 200 mg/l)	296	338	423	328	427
$\Delta\gamma$ (mg/l) (sample + addition) − (sample)	139	136	neg.	6	121

in the urine. The levels of albumin were 10–20 × higher than the threshold value for all samples, indicating the possibility of renal complications in diabetic patients.

In addition, the samples were further spiked with an additional constant amount of albumin providing a theoretical increase of concentration of 200 mg/l. Thus modified samples showed decrease of signal and increase of albumin level (Table III). For the originally high-level samples no. 8 and 9, the addition of albumin was without any effect; the assay was strongly affected by the presence of high concentration of non-specifically bound urine compounds. A similar problem was observed in the last sample exhibiting alkaline pH, where the measured concentration of albumin did not correlate to the concentration of protein. The effect of pH on the assay was negligible in the presence of the working buffer (pH 7).

The total time of analysis was around 16 min including 5 min preincubation, 10 min measurement and 1 min regeneration. The results of the repeated individual measurements of the same sample (Fig. 7: 4.41, 4.14, 3.95, 4.92 and 4.77×10^{-4}) provided the mean value of 4.43×10^{-4} (RSD = 9.22%), which seems satisfactory for the immunosensor based on the SPR sensor Spreeta and considering different sensors used on different days.

CONCLUSION

The SPR sensor with immobilized HSA was employed for the competitive assay of albumin in urine. The measuring range was between 0.1 and 10 mg/l. The normal concentration of albumin in urine is up to 20 mg/l; therefore, using a suitable dilution of urine samples it was possible to determine low concentrations of albumin as well as reliably identify substantially increased levels in diabetic patients. The direct assay of HSA with immobilized antibody was studied too, but without any positive results. Non-specific interaction of urine components with HSA immobilized on the sensing surface remains a problem negatively affecting the performance of the developed assay. This problem was partially solved using the differential measurement of the urine sample alone and in the mixture with antibody. Another possibility might be a dual channel system for instant compensation of the non-specific interactions. The main advantage of the SPR Spreeta sensor was the possibility to observe immunoaffinity interactions in real time without any labeling and, hence, without any destruction of sample.

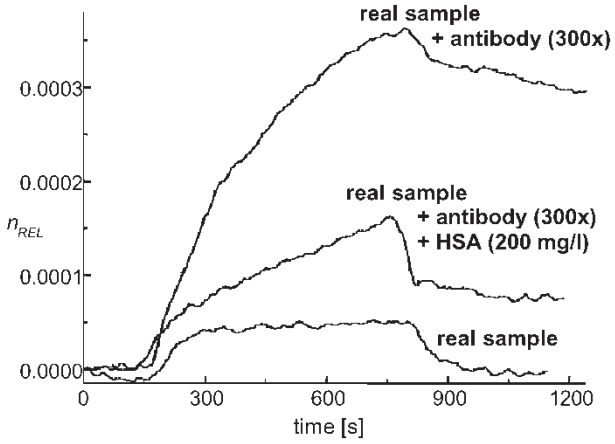

FIGURE 6 The real traces from the Spreeta immunosensor analysis for the urine sample no. 6 from Table III (100 × diluted) in the absence and presence of antibody (ascites fluid, 300-times diluted). In addition, the trace for the mixture containing the sample, antibody and added albumin (increase of concentration of 200 mg/l) is shown.

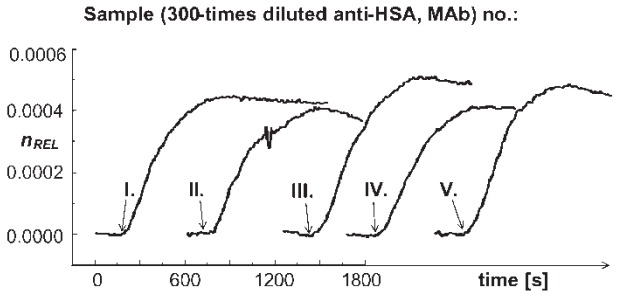

FIGURE 7 Repeated analysis of the same sample containing 300-times diluted anti-HSA MAb using two different sensors on different days. Responses no. I and II were measured using sensor no. 1 on different days (I on day 1, II on day 2). Responses no. III, IV and V were measured using sensor no. 2 (III and IV on day 1, V on day 2).

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