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ORIGINAL PAPER

Surface plasmon resonance aided electrochemical immunosensor for CK-MB determination in undiluted serum samples

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Abstract This article presents a simple chronoamperometric immunosensor for the quantitative assessment of creatine kinase MB (CK-MB) in 50 μL undiluted serum samples. The immunosensor consists of gold working and counter electrodes patterned onto a glass chip by thin-film photolithography and an external Ag|AgCl reference electrode. The detection limit (DL) of the chronoamperometric method is 13 ng mL^{-1} (DL = 2×RMSD/S, where RMSD is the residual mean standard deviation of the measured points around a calibration curve with a slope of S). In spiked serum samples, the response was linear up to 300 ng mL^{-1} of CK-MB. A surface plasmon resonance (SPR) system with simultaneous electrochemical detection (EC-SPR) aided the development of the sandwich immunoassay. Real-time monitoring of the SPR signal was used to optimize the capture antibody immobilization, CK-MB and detection antibody binding, as well as to minimize the nonspecific adsorption of serum proteins to the sensor surface. The detection antibody has been labeled with alkaline phosphatase (ALP) enzyme for sensitive electrochemical detection. ALP catalyzes the hydrolysis of ascorbic acid phosphate and generates ascorbic acid, which is measured chronoamperometrically. The electrochemical immunoassay for CK-MB was less sensitive to nonspecific adsorption related interferences, had a better detection limit, and required a lower volume of sample than the SPR method.

Keywords Creatine kinase MB · Immunosensor · SPR · Electrochemistry · Serum

Introduction

Early and quick diagnosis of acute myocardial infarction (AMI) can save lives and reduce costs in patient treatment [1–4]. Biochemical markers of cardiac injury play an essential role in the diagnosis, prognosis, monitoring, and risk stratification of suspected heart attack patients [1–3]. In addition, they are fundamentally important in therapeutic and interventional guidelines used by clinicians [1]. The development of commercial assays for the determination of cardiac proteins is considered one of the most important innovations in the field of cardiovascular diagnostics in the last decade [3]. The number of papers published on cardiac proteins and their clinical relevance has exploded in recent years. *Clinica Chimica Acta* dedicated a special issue to how biochemical markers have improved cardiovascular disease management. In response to the developments in the field, new recommendations were proposed for the use of cardiac markers in coronary artery diseases [5], and a consensus document was devised for the redefinition of myocardial infarction [6].

Since it became clear that reducing the time-to-treatment of patients with suspected myocardial injury leads to better outcomes, the National Heart Attack Alert program recommended that physicians should treat patients within

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30 min of arrival in emergency departments. To expedite treatment and improve outcomes, tests with shorter turnaround times and point of care testing (POCT) devices are needed. The rationale for using POCT of cardiac proteins has been justified in large-scale studies [1, 7–9]. Unfortunately, commercial POCT devices commonly do not produce the same results as the central laboratory analyzers, which is a requirement for consistent clinical interpretation of the measured values [4, 10].

The measurement of certain cardiac proteins like creatine kinase MB (CK-MB) or cardiac troponins (cTnI and cTnT) in blood or serum samples provides a convenient pathway for diagnosis and guides the therapy of AMI [1–3]. The concentrations of these proteins in blood depend on the severity of AMI and the delay time after the onset of AMI [2, 3, 10, 11]. The existing POCT devices for cardiac proteins generally employ immunochromatographic separation and spectrophotometric or spectrofluorometric detection systems [12, 13]. However, immunoassays with electrochemical detection are gaining popularity as a way to simplify the technology [14–16].

Surface plasmon resonance (SPR) is a surface-sensitive optical technique typically used to quantify the amounts of adsorbed species on gold surfaces [17]. SPR is one of the few real-time and label-free methods used to track biorecognition events [18]. Moreover, it is a versatile and sensitive technique that can be made selective through the immobilization of specific reagents, such as antibodies, onto the sensing surface of the SPR chips. SPR-based immunosensors are appealing because of their sensitivity for high molecular weight analytes as well as their outstanding specificity due to antigen–antibody immunoreactions. However, the SPR-based methods are also very sensitive to nonspecific adsorption related interferences. Consequently, SPR is an excellent method for studying and minimizing nonspecific adsorption related interferences in complex matrices [18, 19]. The detrimental effect of such interferences in SPR-based methods can be partially offset by employing a reference channel [20] and utilizing the difference between the SPR signals in the sample and reference channels as analytical information.

Incorporating labels (e.g., nanoparticles) can improve the attainable detection limit (DL) of SPR-based methods [21, 22]. SPR-based immunosensors have shorter response times and can have better detection limits than conventional ELISAs. Due to these valuable features, the importance of SPR-based methods and sensors has rapidly increased [17].

Electrochemical and SPR methods have complementary advantages, but the simultaneous application of amperometric and SPR responses presents some hurdles. The functionalized thiol monolayers that are commonly utilized for covalent immobilization of biomolecules onto the SPR chip surfaces can block the charge transfer reaction on the gold surface when it is used for simultaneous amperometric detection [23–25]. However, such problems can be eliminated or minimized by using short alkyl chain thiols, like thioctic acid (ThA), for the immobilization [24–27].

This study presents the development of an electrochemical immunosensor for the quantitative assessment of CK-MB in untreated serum samples. A schematic of the differential cell of the SPR instrument with the possibility of simultaneous electrochemical detection (EC-SPR) is shown in Fig. 1A. The SPR instrument was used to optimize the steps of a sandwich immunoassay with alkaline phosphatase labeled detection antibody. The resulting protocol was then adapted to amperometric microcells requiring only 50 μL of serum for each assay. Microfabricated planar electrodes were patterned at the bottom of these microcells, with the same dimensions as individual wells in a 96-well microtiter plate. The CK-MB concentrations were measured in six wells simultaneously. Figures 1B and C show a schematic drawing of an electrochemical microcell and a photograph of six electrochemical cells, respectively.

Since CK-MB is no longer considered the gold standard chemical marker for myocardial infarction, the protocol described in this paper for CK-MB is only the first step in the development of a multi-analyte electrochemical immunoassay in which cardiac troponin I (cTr-I) and cardiac troponin T (cTr-T) will be determined at the same time as CK-MB levels by utilizing three spatially separated working electrodes in the same electrochemical microcell. The individual working electrode surfaces will be specifically patterned with capture antibodies for CK-MB, cTr-I and cTr-T. Each assay will use ALP-labeled detection antibodies. A potential planar electrochemical cell design for multi-analyte electrochemical detection was published recently [27].

Experimental

Apparatus

Electrochemical experiments were performed with an EcoChemie (Utrecht, The Netherlands) PGSTAT12 potentiostat/galvanostat controlled by the GPES 4.9.5 software package. SPR measurements were carried out with an Autolab ESPRIT system (also from EcoChemie), which is equipped with an electrochemical cell for simultaneous SPR and electrochemical detection (Fig. 1A). For the temperature control of the SPR instrument, a Lauda Ecoline (Lauda-Königshofen, Germany) model RE206 thermostat was used. Microfabricated chips were cleaned in a Harrick Scientific (Pleasantville, NY, USA) PDC-32G model plasma cleaner that was connected to a Robinair, CoolTech (Owatonna, MN, USA) high-performance vacuum pump.

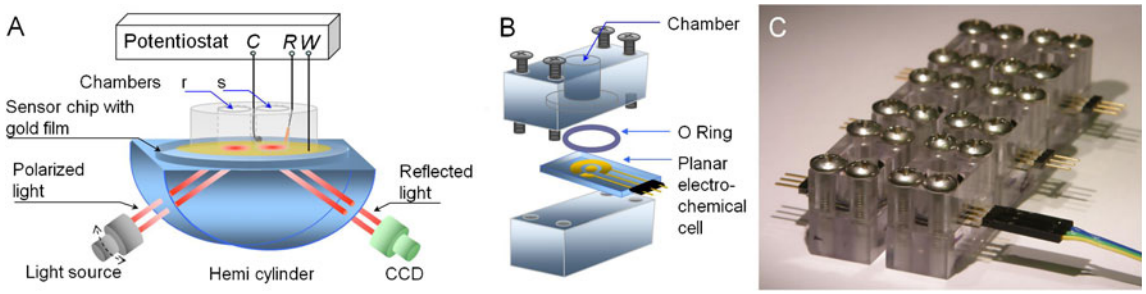

Fig. 1 SPR and electrochemical cells utilized in this study. **A** Differential SPR detector cell for simultaneous SPR and electrochemical detection. **B** Electrochemical microcell with a planar sensor chip fixed to the bottom of the sample well. **C** Photograph of six electrochemical microcells for simultaneous electrochemical assays. *C*, counter electrode; *R*, reference electrode; *W*, working electrode; *r*, reference chamber; *s*, signal chamber

Additional equipment included a touch mixer model 232 from Fisher Scientific (Fair Lawn, NJ, USA) to homogenize spiked serum samples, an Eppendorf model 5415C centrifuge from Brinkmann Instruments (Riverview, FL, USA), and an Orion model 720 A pH/mV meter (MA, USA).

Electrodes

Electrochemical measurements were performed in conventional three-electrode cells. In the Autolab ESPRIT system, the SPR gold disc served as the working electrode, while a platinum wire was used as counter electrode. The microfabricated planar electrochemical cells were provided by Aegis Technologies Group, Inc. (Huntsville, AL, USA). They were patterned on 1.0 cm×1.5 cm glass chips with standard thin-film photolithography using a 1.0 μm thick polyimide insulation layer. The electrochemical surface areas of the working and counter electrodes were 1.46 mm^2 and 13.4 mm^2, respectively. These chips were fixed to the bottom of polycarbonate chambers (6.40 mm diameter, 12.2 mm deep), as shown in Fig. 1B. The immunoassays were performed in these chambers. During the chronoamperometric measurements an external Ag|AgCl|NaCl (0.1 M) served as reference electrode.

Materials

Chemicals All solutions were prepared with 18 MΩ cm^{-1} deionized water from a Millipore (Bedford, MA, USA) Milli-Q system. H_2SO_4 98%, H_2O_2 30%, *N*-hydroxysuccinimide (NHS), glacial acetic acid, Na_2HPO_4, KH_2PO_4, HCl, NaOH, H_3BO_3, and NaCl were purchased from Fisher Scientific (Fair Lawn, NJ, USA). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) 99%, ethanol HPLC grade, ascorbic acid (AA), thioctic acid (ThA), ascorbic acid 2-phosphate (AAP), bovine serum albumin (BSA), and $MgCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human creatine-kinase MB isoenzyme type 2 (CK-MB) was purchased from Lee Biosolutions Inc. (St. Louis, MO, USA). Detection (Ab404) and capture (Ab19603) monoclonal antibodies against human creatine-kinase MB were purchased from Abcam (Cambridge, MA, USA). Alkaline phosphatase (ALP) and conjugation reagents for labeling the Ab404 antibody were bought from Innova Bioscience (Cambridge, UK). Liquichek CK-MB isoenzyme controls and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). SPR discs were purchased from Xantec Bioanalytics (Dusseldorf, Germany). Super-blocking buffer (SBB) and fish serum (SEA) blocking buffer were products of Thermo Scientific (Waltham, MA, USA). Ultrafiltrated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Ultrahigh-purity argon was obtained from Nexair (Memphis, TN, USA).

Buffers and reagents PBS pH 7.4 (0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) was freshly prepared every two weeks. PBST buffers were prepared with 0.5% (w/v) and 0.05% (w/v) Tween-20. These buffers are labeled as PBST with the Tween 20 concentration in parentheses, e.g., PBST (0.5%). Tween-20 was also added to SBB and SEA blocking buffers in 0.05% (w/v) concentration. Acetic acid (HAc) buffer was prepared by adding concentrated NaOH to 10 mM HAc to set the pH to 4.5. The pH of the borate buffer (10 mM H_3BO_3, 0.1 M NaCl, and 10 mM $MgCl_2$) was set to 9.5 with NaOH. Standards of 20 mM AA and 20 mM AAP were prepared in pH 9.5 borate buffer. Solutions of 0.1 M HCl and 0.1 M NaOH were used for SPR disc stabilization. ThA (5 mM) was dissolved in ethanol with 5% HAc (5% EtHAc). Concentrated HCl was used to set the pH of 1 M ethanolamine ($EtNH_2$) to 8.0. Solutions of 50 mM EDC and 50 mM NHS were freshly prepared in pH 4.5 HAc buffer. All solutions were stored at 4 °C until use.

Protein solutions A 2.5% (w/v) BSA solution was prepared in PBST (0.05%) and stored at −80 °C in 0.5 mL aliquots

until use. CK-MB solution was diluted to 10 μg mL^{-1} with PBST (0.05%) and stored at −80 °C in 10 μL aliquots. Capture antibody (Ab19603) solution was divided into aliquots and stored at −20 °C. Before immobilization, it was diluted to 50 μg mL^{-1} with acetate buffer. Detection antibody (Ab404) was conjugated with ALP, separated into aliquots and stored at −20 °C. Aliquots of the detection antibody were thawed and diluted with PBST (0.05%) before use.

Preparation of SPR chips First, gold-coated SPR discs were cleaned in a 2:1 mixture of 98% H_2SO_4 and 30% H_2O_2 (piranha solution) for 40 min. Next, the discs were thoroughly rinsed with water, followed by ethanol. After cleaning, the chips were immersed in 5 mM ThA in 5% EtHAc and incubated for 72 h. The thioctic acid SAMs prepared in the presence of 5% acetic acid containing ethanol were reported to be well organized [26]. After incubation, SPR discs were thoroughly rinsed with 5% EtHAc and dried with argon. The ThA-coated chips were used within one week of preparation.

Preparation of the amperometric chips Planar amperometric cells were cleaned for 3 min with radiofrequency air plasma (18 W) at 300 mTorr pressure. The cleaned chips were assembled in polycarbonate chambers (6.40 mm diameter, 12.2 mm deep) and immediately exposed to 200 μL of 5 mM ThA solution. The chambers were covered with parafilm and stored in the hood for 72 h.

Stabilization of the SAM Before the capture antibody immobilization, the ThA SAM-coated gold surfaces were stabilized according to the protocol shown in Fig. 2A. First the surface was washed with 5% EtHAc for 20 min (step I). During this washing process, the cell was filled and drained with fresh solution every 100 s to remove unbound thioctic acid molecules and to achieve an ordered SAM [26]. Next, the surface was charged and uncharged by alternate exposure to 0.1 M HCl and 0.1 M NaOH solutions (step II). These washing steps gradually replace ethanol with water and dissolved ions while the surface approaches steady state [28]. Finally, the surface was washed with a pH 4.5 acetate buffer (step III).

The solution was replaced every 5 min. After 50 min of washing, a stable SPR signal was obtained (i.e., the standard deviation of the differential signal was 0.18 m° throughout a 30 min period without replacing the buffer solution).

Capture antibody immobilization EDC/NHS coupling was used to immobilize the capture antibody onto the ThA SAM [29]. In previous reports, acetate buffers [30], water [31], and diverse phosphate buffers [23, 32] were used during the conjugation of carboxyl groups to primary

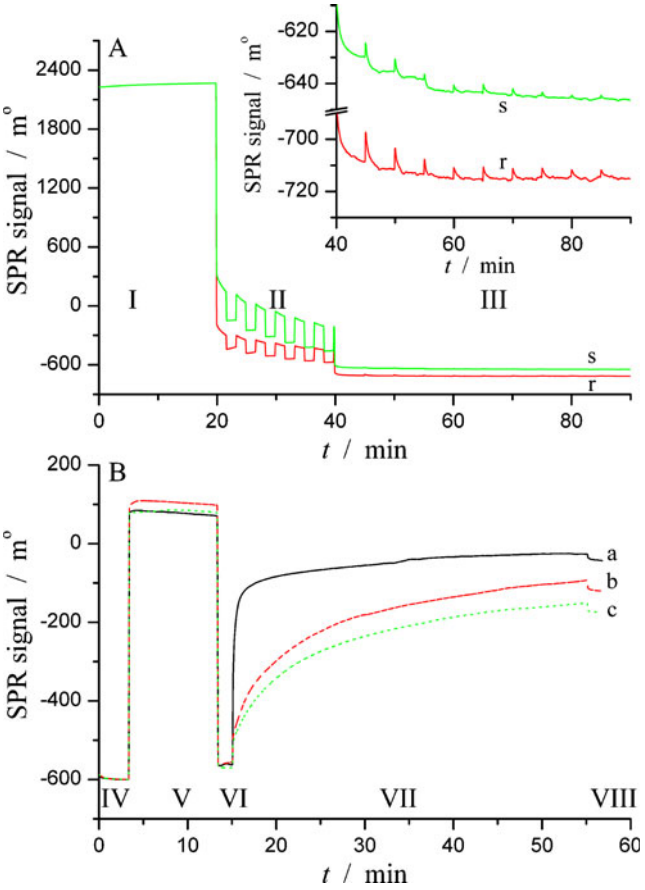

Fig. 2 **A** Traces of SPR angle shifts for reference (*r*) and signal (*s*) channels during the stabilization process of a thioctic acid SAM. Washing steps: I, 5% EtHAc solution; II alternating between 0.1 M HCl and 0.1 M NaOH (6 cycles); III, acetate buffer pH 4.5 (refreshed every 5 min). *Inset* shows a magnification of step III. **B** SPR angle shifts during the immobilization of the anti-CK-MB capture antibody (Ab19603) by EDC/NHS chemistry in base electrolyte (BE): *a*, acetate buffer pH 4.5; *b*, PBS pH 7.4; *c*, 0.1 M NaCl pH 7.1; IV, BE; V, EDC/NHS in BE; VI, washing with BE; VII, incubation of 50 ppm Ab19603 in BE; VIII, washing with BE

amines. Figure 2B summarizes the SPR transients when the immobilization of capture antibody is performed in different background electrolytes. First, the SPR chip surface was activated for 10 min in 25 mM EDC and 25 mM NHS mixture (step V). Then the surface was washed with the tested base electrolyte and exposed to 50 μL of 50 μg mL^{-1} capture antibody for 40 min (steps VI and VII). Finally, the surface was washed again with base electrolyte (step VIII). In the presence of pH 4.5 acetate buffer, the surface coverage was estimated as ~70% based on the sensitivity of the SPR instrument (120 m° $ng^{-1}mm^{-2}$) and on the assumption that an individual antibody occupies a surface area of 47 nm^2 [33, 34].

Conjugation For the conjugation of detection antibody (Ab404) with ALP, a protocol provided by Innova Bioscience was followed. The pH of the detection antibody

solution was raised to pH ~7.4 by adding 29 μL of 0.4 M Na_2HPO_4 to its stock solution. Next, 10 μL of modifier was added and mixed with the antibody and the lyophilized ALP was gently resuspended in this solution. The suspension was kept at room temperature for 4.5 h. Subsequently, 10 μL of quencher was added to the mixture. After 30 min, the modified detection antibody was separated into aliquots and stored at −20 °C.

Preparation of human serum samples The Central Laboratory of the Methodist University Hospital (Memphis, TN, USA) provided pooled serum. The CK-MB concentration in the pooled serum was (8.1±0.7) ng mL^{-1}. It was determined with a Beckman Coulter UniCel Dxl 800 clinical analyzer. The pooled serum was homogenized, divided into aliquots of 120 μL, and stored at −80 °C. Before use, the samples were thawed and spiked with different amounts of CK-MB dissolved in 30 μL PBST (0.5%). The same protocol was followed with CK-MB isoenzyme controls.

Results and discussion

In general, the DL of a sandwich immunoassay is determined by the affinity and selectivity of the antibodies, the sensitivity of the detection method, and interferences related to nonspecific adsorption. Nonspecific adsorption represents a major obstacle for sensors intended for the analysis of complex biological matrices [18, 19]. Although a large variety of surface modifications and treatments are available, there is no general protocol to minimize the detrimental effects of nonspecific adsorption for all applications. SPR is an ideal tool for studying nonspecific adsorption on surfaces. To find the most suitable blocking solution against nonspecific adsorption, SPR chips were treated with different blocking solutions; see Fig. S1 in the "Electronic supplementary material".

Surfaces blocked with undiluted SBB presented the highest nonspecific adsorption, while the lowest nonspecific adsorption was found for surfaces treated with FBS. However, even with FBS as blocking buffer, the nonspecific adsorption related angle shift (~60 m°) is rather high [35]. Thus, only part of the nonspecific adsorption related changes can be removed by considering a differential signal. Based on these preliminary data, complete SPR immunoassays were performed using pooled human serum samples spiked with CK-MB standards. In these sandwich immunoassays, the blocking buffer was composed of 10% SBB+0.2 M $EtNH_2$+ 1.25% BSA in PBST (SEtBSA), or was realized by the addition of 10% SEA in PBST and then 1M $EtNH_2$ (SEAB). These blocking solutions minimized the nonspecific adsorption of the ALP-labeled detection antibody and provided similar SPR angle shifts in the reference and signal channels when they were incubated with serum samples (Fig. 3).

SPR immunoassay protocol

Figure 3 shows the entire SPR-based protocol for CK-MB quantification. The traces labeled as (s, red), (r, black), and (d, blue) are the angle shifts recorded in the signal (s), reference (r), and differential (d) channels of the instrument, i.e., curve (d) represents the difference in the angle shifts between the (s) and (r) channels. The steps of the protocol were the same for the signal and reference channels (the same chemicals were introduced into the signal and reference chambers of the instrument), except for the capture antibody immobilization step. A scheme of the chemical steps involved is presented in Fig. 3B. Following the stabilization of ThA SAM, its activation with EDC/NHS and a washing step with pH 4.50 HAc, 50 μL of the capture antibody solution (50 μg mL^{-1} in pH 4.5 HAc) were dispensed into the signal channel chamber. However, only HAc pH 4.5 was added to the reference channel (step II). The immobilization of the capture antibody induced a (450±50) m° angle shift in the SPR signal channel after washing with pH 4.5 acetate buffer, while the reference channel signal remained practically unchanged (< 5 m°).

In the next step of the protocol, acetate buffer was replaced by PBS and the surfaces treated with the blocking solution (step III). Subsequently, the blocked surfaces were washed with PBS and PBST (0.5%), incubated with the serum sample (step IV), and washed again with PBS and PBST (0.5%). In the final step, the chambers were filled with 50 μL of a solution of detection antibody (50 μg mL^{-1} dissolved in PBST (0.05%)), incubated for 30 min (step V), and washed with PBS. The difference between the SPR signals recorded before and after the incubation of the detection antibody provided the analytical signal for the quantification of CK-MB. As shown in Fig. 3A, the introduction of the ALP-labeled detection antibody did not generate a significant SPR angle shift in the reference channel (also refer to Fig. S2 in the "Electronic supplementary material").

SPR calibration curves

Figure 4 shows differential SPR signals corresponding to the binding of ALP-labeled detection antibody to SPR immunosensor chips. The individual traces represent binding curves recorded with distinct SPR chips. Before the detection antibody binding step, the chip surfaces were blocked with SEtBSA and subsequently exposed to human serum samples with different CK-MB concentrations. The inset displays a calibration curve constructed from these

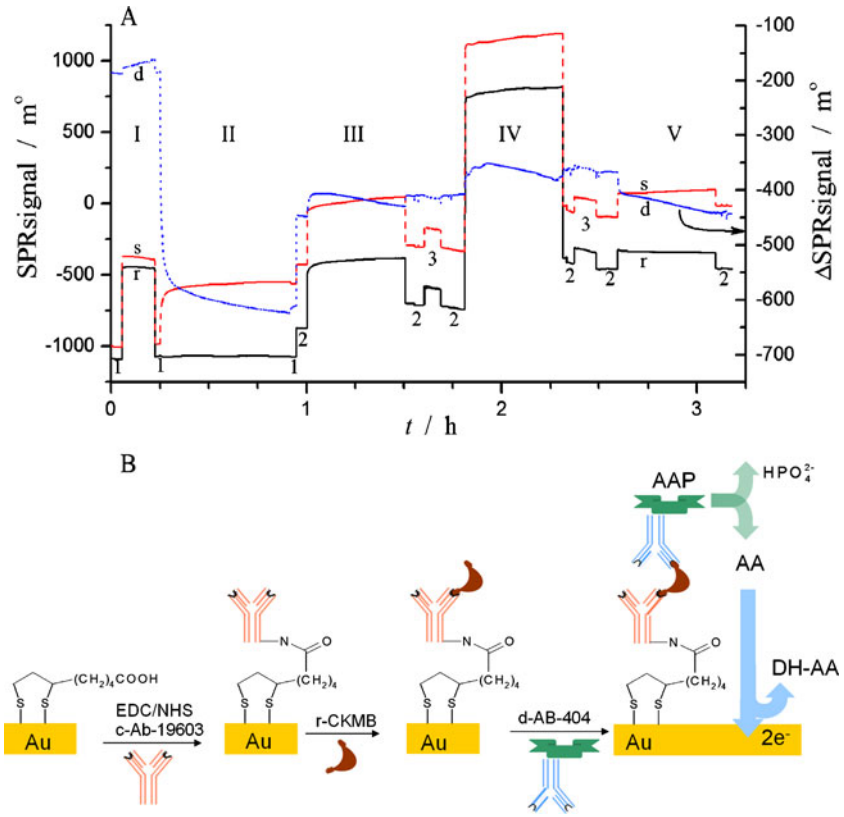

Fig. 3 **A** SPR angle shifts of the signal (s), reference (r) and differential (d) channels during a complete sandwich immunoassay for CK-MB quantification. I, Surface modification with EDC/NHS; II, immobilization of the capture antibody; III, blocking with SEAB; IV, incubation with human serum; V, binding of ALP-labeled detection antibody. Washing steps: 1, HAc pH 4.5; 2, PBS; 3, PBST (0.5%). **B** Scheme of the chemical steps involved and the electrochemical detection

transients. Least squares linear regression analysis of the calibration data provided the following equation:

$$\Delta SPR_{\mathrm{SEtBSA}}(\mathrm{m}^\circ) = (-4 \pm 4) + (0.69 \pm 0.09) C_{\mathrm{CK-MB}} \quad (1)$$

Similar SPR transients were recorded using SEAB as blocking buffer (not shown). The equation of the corresponding calibration curve is:

$$\Delta SPR_{\mathrm{SEAB}}(\mathrm{m}^\circ) = (38 \pm 4) + (0.95 \pm 0.08) C_{\mathrm{CK-MB}} \quad (2)$$

The two calibration curves were of comparable quality. The residual mean standard deviations (RMSDs) of the measured points around the fitted lines were 7.6 m° and 7.3 m°, respectively. Although the slope of the calibration curve with SEAB as blocking buffer was larger, SEtBSA was preferred in the SPR experiments. With SEtBSA used as blocking buffer, the detection antibody had very low nonspecific adsorption in both channels and the calibration curve had an intercept that was close to zero.

Reproducibility of the SPR-based immunoassay for CK-MB

To assess the detection limit of the SPR-based immunoassay, the reproducibility of the SPR signal in pooled human serum samples was determined. The detailed protocol and a figure are provided in the "Electronic supplementary material."

The standard deviation of the SPR differential signal ($s_{\mathrm{d,d}}$) is4.4 m°. If $s_{\mathrm{d,d}}$is used in combination with the IUPAC recommendation for assessing the detection limit ($\mathrm{DL} = 3 \times s_{\mathrm{d}}/\mathrm{sensitivity}$), DL=14 ng mL^{-1} and DL=

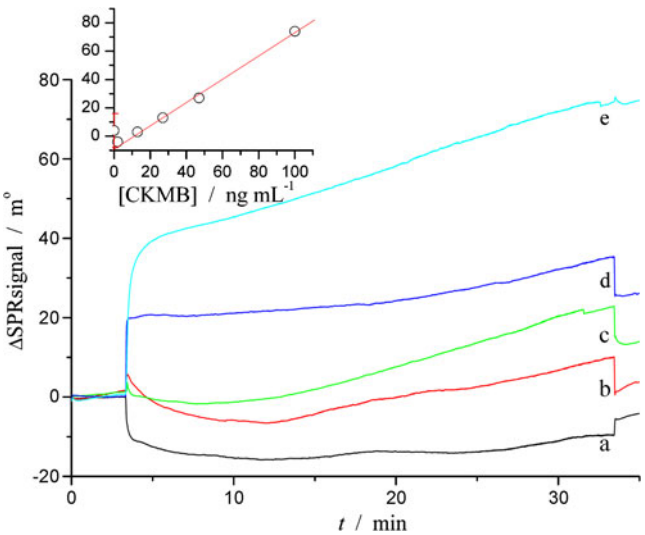

Fig. 4 Time-dependent changes in the differential SPR signal during the exposure of the SPR chip surfaces to the ALP-labeled detection antibody solution. The chips were previously incubated with CK-MB-spiked human serum samples. Concentrations of CK-MB added to the serum samples (in ng mL^{-1}): a, 2; b, 13; c, 27; d, 47; e, 100. Blocking buffer: SEtBSA. *Inset*: calibration curve generated from the ΔSPR shifts after 30 min of incubation, $\mathrm{RMSD}_{\mathrm{SEtBSA}}$=7.6 m° ($r^2$=0.93718)

19 ng mL^{-1} are obtained for the assays with SEAB and SEtBSA blocking buffers, respectively. Using the RMSD values of the data points around the fitted calibration curves (7.3 and 7.6 m°) in combination with the formula DL = 2×RMSD/S (where *S* is the slope of the calibration curve) yields similar detection limits for the experiments with SEAB and SEtBSA as blocking buffers: DL= 15 ng mL^{-1} and DL=22 ng mL^{-1}, respectively.

These detection limits are about two orders of magnitude higher than the instrumental detection limit, which is calculated from the standard deviation of the baseline noise (0.18 m° in our experiments). Reporting the instrumental detection limit as the detection limit of the SPR immunoassay would be misleading, since it does not consider all sources of errors that may contribute to the random scattering of the SPR angle shift of an immunoassay where each measurement is performed on a new sensor chip. Unfortunately, instrumental signal-to-noise ratios are commonly used to report the DLs of SPR-based immunoassays [35–39].

Electrochemical detection subsequent to SPR-based assays

In electrochemical immunoassays, the label enzyme activity is measured as a function of the analyte concentration. The enzyme activity can be determined through the quantitative assessment of an electrochemically active product generated in the enzyme-catalyzed reaction. In this work, ascorbic acid phosphate (AAP) was used as the substrate for the ALP enzyme, and the rate of generation of ascorbic acid (AA) provided the analytically relevant signal [40]. Cyclic voltammetry was used to determine the optimal voltage for the chronoamperometric determination of AA. In our protocol, once the SPR immunoassay was finished, the PBS solution was replaced by pH 9.5 borate buffer to provide an optimal pH for the ALP enzyme. AAP was added to the chamber 3 min after the application of 0.5 V vs. Ag/AgCl reference electrode in the SPR cell. Figure 5 shows the chronoamperometric traces recorded with SPR sensor chips in the ALP-labeled sandwich immunoassays using SEtBSA as blocking buffer. The calibration curve constructed from these chronoamperometric transients is shown as an inset. The parameters of the fitted line were determined by least squares linear regression analysis:

$$I_{\mathrm{SEtBSA}}/\mu\mathrm{A} = (0.17 \pm 0.02) + (74 \pm 6)10^{-4} C_{\mathrm{CK-MB}}. \quad (3)$$

The chronoamperometric transients were similar when SEAB was used as blocking buffer, but the corresponding calibration curve had a larger slope and a smaller offset:

$$I_{\mathrm{SEAB}}/\mu\mathrm{A} = (0.10 \pm 0.04) + (16 \pm 1)10^{-3} C_{\mathrm{CK-MB}}. \quad (4)$$

This difference in sensitivity may be related to the slower diffusion of AA through the SEtBSA-blocked surface. The

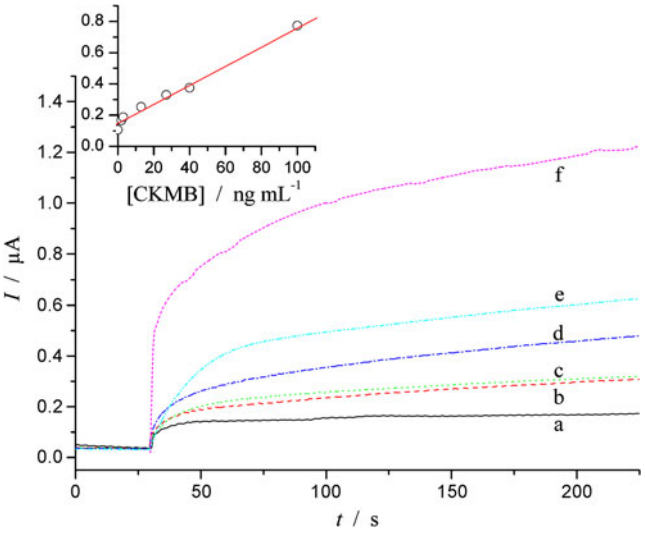

Fig. 5 Chronoamperometric profiles recorded with SPR sensor chips in ALP-labeled sandwich immunoassays. The traces correspond to the oxidation of AA generated from AAP. Applied potential: 0.5 V vs. Ag|AgCl. Background electrolyte: 60 µL, 0.1 M borate buffer pH 9.5+60 µL, 20 mM AAP. CK-MB concentrations in spiked human serum samples (ng mL^{-1}): a, 0; b, 2; c, 3; d, 13; e, 40; f, 100. Blocking buffer: SEtBSA. *Inset*: calibration curve constructed from the current values at 60 s after AAP addition, $RMSD_{SEtBSA}$=0.050 µA (r^2=0.97158)

detection limits calculated from the RMSD values of the data are DL_{SEtBSA}=14 ng mL^{-1} and DL_{SEAB}=8 ng mL^{-1}. These DL values are approximately a factor of two lower than those achieved with the SPR-based immunoassay.

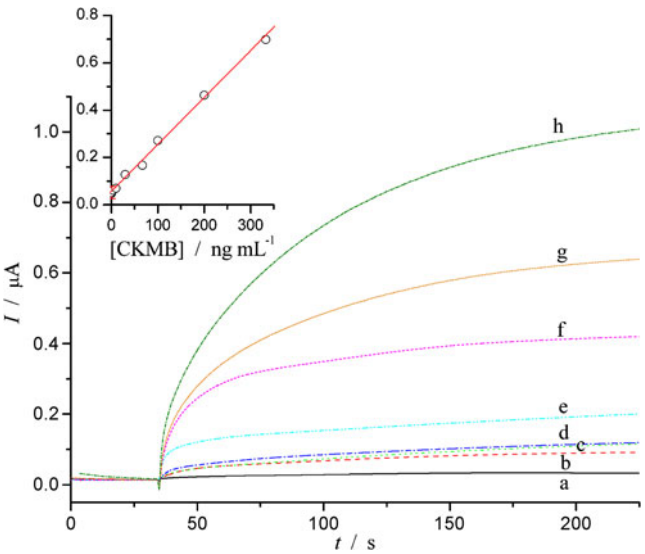

Fig. 6 *I–t* profiles recorded with planar electrochemical sensor chips in ALP-labeled sandwich immunoassay. The traces correspond to the oxidation of AA at 0.5 V vs. Ag|AgCl. Background electrolyte involved 60 µL of 0.1 M borate buffer pH 9.5. After the delay time, 60 µL of 20 mM AAP were added. Concentrations of CK-MB added to serum samples (ng mL^{-1}): a, FBS; b, 0; c, 3; d, 10; e, 30; f, 100; g, 200; h, 333. Blocking buffer used in the assay: SEtBSA. *Inset*: calibration curve constructed from the current values at 60 s after the addition of AAP; RMSD=0.014 µA (r^2=0.99658)

Single-use, planar amperometric cell for CK-MB determination in serum

The optimized immunoassay protocol was applied to planar electrochemical cells with gold working and counter electrodes fabricated by thin-film photolithography on glass chips. These glass chips were fixed at the bottom of polycarbonate chambers with dimensions identical to the individual wells of a 96-well microtiter plate (Fig. 1B). An AgCl-coated silver wire immersed into the sample solution served as the reference electrode. Six chambers were employed for the simultaneous analysis of six serum samples (Fig. 1C). Figure 6 shows a set of chronoamperometric curves corresponding to the detection step of the CK-MB immunoassay in human serum. Each curve represents a measurement on a single-use chip. The chips were incubated with pooled serum samples spiked with different levels of CK-MB. In the assays, SEtBSA was used as blocking buffer and FBS was employed as blank (curve a). The calibration curve (Fig. 6, inset) merges the current values measured 60 s after the addition of AAP. The parameters of the calibration curve were determined by least squares linear regression analysis:

$$I/\mu\mathrm{A} = (0.069 \pm 0.006) + (20 \pm 1)10^{-4} C_{\mathrm{CK-MB}}. \qquad (5)$$

The lower sensitivity compared to the electrochemical immunoassay performed in the EC-SPR instrument (Fig. 5 and Eq. 3) is in agreement with the smaller surface area of the working electrode. The smaller sensitivity coincides with a smaller RMSD value (0.014 μA). Accordingly, the planar amperometric cells provided a DL=13 ng mL^{-1}, which is very similar to the detection limit of the EC-SPR system. With manual handling, six assays could be performed in two hours.

Conclusions

This study presents the SPR-aided development of an amperometric immunosensor for the detection of CK-MB in undiluted human sera. The calibration curve developed from the amperometric responses of individual chips in human serum samples (each sample was measured with a different chip) was linear up to 300 ng mL^{-1} CK-MB. The detection limit of the chronoamperometric method was determined as 13 ng mL^{-1} using the formula DL = 2×RMSD/S. The CK-MB levels can increase from (4±2) ng mL^{-1} to values as high as 400 ng mL^{-1} as a consequence of severe myocardial injury [2, 11]. Although this DL is higher than the CK-MB levels in healthy individuals, the sensor could be used to quantify CK-MB in pathological samples.

By comparing the performance of our SPR with those of electrochemical-based CK-MB immunoassays, the following general conclusions can be drawn. (i) The SPR immunosensor was inadequate for use in "label-free mode" to measure physiologically relevant levels of CK-MB. (ii) The DL of the sandwich immunoassay with electrochemical detection was half that achieved with SPR detection in the same assay. (iii) Electrochemical detection was found to be less sensitive to nonspecific adsorption related interferences than SPR-based detection in sandwich immunoassays. Differential SPR measurements barely reduced these interferences due to unavoidable discrepancies between the sensor and reference channel surfaces. (iv) SPR measurements provide an ideal tool for minimizing the nonspecific adsorption during assay development. (v) Microfabricated electrochemical cells required only 50 μL of sample to quantify the amount of CK-MB in human serum.

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