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Immunoassay of C-reactive protein by hot electron induced electrochemiluminescence using integrated electrodes with hydrophobic sample confinement

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ABSTRACT

C-reactive protein (CRP) was determined in the concentration range 0.01–10 mg L^{-1} using hot electron induced electrochemiluminescence (HECL) with devices combining both working and counter electrodes and sample confinement on a single chip. The sample area on the electrodes was defined by a hydrophobic ring, which enabled dispensing the reagents and the analyte directly on the electrode. Immunoassay of CRP by HECL using integrated electrodes is a good candidate for a high-sensitivity point-of-care CRP-test, because the concentration range is suitable, miniaturisation of the measurement system has been demonstrated and the assay method with integrated electrodes is easy to use. High-sensitivity CRP tests can be used to monitor the current state of cardiovascular disease and also to predict future cardiovascular problems in apparently healthy people.

1. Introduction

Hot electron induced electrochemiluminescence (HECL) is a method which utilises hot electrons. The energy of these hot electrons is higher than the Fermi energy of the surrounding phase, or higher than the thermal energy of the surrounding phase. In the present method, hot electrons are generated by tunnel emission through a thin insulator film of a conductor/insulator/electrolyte (C/I/E) tunnel junction electrode to an aqueous electrolyte solution during a cathodic pulse. If the energy of the hot electrons is higher than the energy of the conduction band edge of water, they may enter the conduction band of water and become rapidly thermalised and finally hydrated (e^-_{aq}). Cathodically generated strongly oxidising species, e.g. hydroxyl and sulfate radicals from added coreactants, are generated during the HECL process [1–7]. Also F^+ and E'_1 centers existing in the insulating oxide layer may act as oxidants [1,8]. The hot and hydrated electrons and the strongly oxidising species can cause oxidation and reduction reactions, which are not achieved at active metal electrodes using traditional electrochemistry [1–7].

HECL is a selective and sensitive method which can be used to determine luminophores, such as rare-earth metal chelates, in nanomolar to picomolar concentration [9]. It is also possible to detect many organic luminophores, such as fluorescein and rhodamine B which cannot be excited by traditional electrochemistry in aqueous solutions [10,11].

Also some oxidants, such as hydrogen peroxodisulfate and peroxodiphosphate can be determined using HECL [12,13].

One of the advantages of the HECL system is its relatively simple instrumentation. Unlike photoluminescence, HECL does not need any external light source which lowers the cost of instrumentation. In addition, it is a very sensitive method which is relatively easy to miniaturise.

In this research, we have studied the possibility of using a microfabricated integrated electrode chip with hydrophobic sample confinement for immunoassay studies by HECL. In this device the working and counter electrodes and the sample confinement are combined on a single chip. The use of integrated electrodes is one possible way to enhance the benefits of miniaturisation of electrochemical devices. It is difficult to reach the sensitivity and reproducibility needed for a real sample analysis by miniaturisation of discrete electrodes [14]. Therefore, integrated electrodes are already used in many analytical applications, like capillary electrophoresis [15] and impedimetric immunosensors [14], for example.

We have chosen CRP as a model analyte for our immunoassay studies because it is a well-known diagnostic marker. The CRP value in a healthy individual is below 10 mg L^{-1}, but it rises quickly during inflammation or tissue injury [16]. During a bacterial infection

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the CRP level rises substantially, but during a viral infection only a marginal increase is seen. Therefore, CRP levels can be used to differentiate infections, which need antibiotic prescription, from self-limiting illnesses [17–19]. CRP tests are especially useful to differentiate pneumonia from acute bronchitis [17]. There are some CRP point-of-care tests commercially available already now [16]. The determination limits of these kinds of tests are usually between 5–200 mg L^{-1} and the result is available in couple of minutes. With the help of these tests, together with the symptoms of the patient, doctors can determine the need for antibiotics already during the consultation, without the need for lengthy laboratory tests [17,20]. Point-of-care CRP testing is also a promising way to reduce unnecessary use of antibiotics [17].

However, the determination limit of most point-of-care CRP tests is not sufficient, when there is a need to measure very low CRP-levels (high-sensitivity CRP). Lower concentrations are needed, when CRP levels are used to predict progressive vascular disease or future cardiovascular events in apparently healthy people. CRP is an independent predictor of future cardiovascular events and it might be an even stronger predictor than the concentration of low-density lipoprotein cholesterol (LDL) [21]. After statin therapy those patients who have lower CRP levels have better clinical outcomes than those with higher CRP levels, regardless of their LDL level achieved by the therapy.

It seems that CRP can be used both as a biomarker of cardiovascular disease and as a method to monitor pharmacological interventions used to prevent and treat cardiovascular disease [22]. American Heart Association and Centers for Disease Control and Prevention (CDC) have endorsed guidelines for CRP concentrations: <1, 1–3, and >3 mg L^{-1}, where the concentrations suggest lower, moderate and high relative risk of incident vascular disease, respectively [22]. Additionally, it has been suggested that high-sensitivity CRP might be a useful surrogate marker for detection of inflammation associated with multiple sclerosis relapses [23].

In this paper, we present an immunoassay method which is rapid, easy to use and it is usable as a high-sensitivity point-of-care CRP analysis system with wide dynamic range.

2. Experimental

2.1. Materials

The chips were fabricated on n-type silicon wafers from Okmetic Ltd., of 0.005–0.018 Ω-cm resistivity and (1 1 1) orientation. The chemicals used during processing were standard semiconductor-grade materials.

The primary antibody was anti-hCRP, Lot 0018728, concentration 1040 mg L^{-1}, obtained from Oy Medix Biochemica Ab, Kauniainen, Finland. It was diluted to 50 mM Tris Saline Azide (TSA) buffer consisting of 50 mM TSA, 0.9% NaCl, 0.05% NaN_3, pH 7.75, adjusted with HCl. The saturation solution consisted of 50 mM TSA, 0.1% bovine serum albumin (BSA) and 6% D-sorbitol. The label in the secondary antibody was Tb(III) chelated by N1-(4-isothiocyanatobenzyl)diethylenetriamine-N1,N2,N3,N3-tetraacetate which was obtained from Wallac Oy Turku, Finland. The hCRP standards were obtained from Scripps, San Diego. Standard dilution buffer consisted of 50 mM TSA, 10% D-sorbitol, 0.5% BSA, 0.12% bovine-gamma-globulin, 0.02% Tween 20. The pH of the buffer was adjusted by HCl to 7.83 and the buffer was filtered by 0.2 μm Acrodisc® Syringe Filter with HT Tuffryn® Membrane. Stock solution of labelled secondary antibody had concentration 0.12 mg L^{-1} and the ratio between the chelate and the antibody was 9.1. Secondary labelled antibody was diluted to a buffer consisting of TSA, 0.5% diethylene triamine pentaacetic acid (DTPA)-cleaned BSA, 0.05% bovine-gamma-globulin, 0.01% Tween 20 and 3.5 mM $CaCl_2$. The pH was adjusted by HCl to 7.5. The buffer was filtered by 0.2 μm Acrodisc® Syringe Filter with HT Tuffryn® Membrane. The measuring buffer was borate–azide buffer consisting of 50 mM $Na_2B_4O_7$, 0.1% NaN_3, pH 7.9 adjusted by 5 M HCl. The buffer was filtered by 0.2 μm Acrodisc® Syringe Filter with HT Tuffryn® Membrane. MilliQ-water was made by Millipore's Synergy®.

All the immunoassay reagents, except the borate–azide buffer, were obtained ready made from Labmaster Ltd.

2.2. Instrumentation

The immunoassay measurements were carried out in a cell compartment, which enables contacts both from front (Pt anode) and backside (Si cathode) of the chip in order to connect it to an in-house built coulostatic pulse generator [24]. Coulostatic pulses of 15 V were applied at a rate of 20 Hz and the electric charge was 12.6 μC per pulse. The data were collected using a time-resolved method to further enhance sensitivity. This procedure efficiently separates the long-lived luminescence from the background luminescence [9]. After every pulse a 50 μs delay was applied before starting the 3 ms data acquisition period. The photon counts were integrated over 300 excitation cycles.

Optical detection was performed with a photomultiplier tube (PerkinElmer MH1993, 1364-H-064) through an interference filter (550 nm filter, bandwidth 40 nm) passing only the strongest spectral line of Tb(III) ($^5D_4 \rightarrow {}^7F_2$) [25]. A separate photon counter (SR400 two channel gated photon counter, Stanford Research System) with an amplifier (DC-300 MHz Amplifier, Stanford Research System) was connected to the photomultiplier.

2.3. Procedures

2.3.1. Silicon device fabrication

The silicon wafers were first RCA-cleaned with SC-1 (NH_4OH/H_2O_2 solution at 80 °C), dilute HF and SC-2 (HCl/H_2O_2 solution at 80 °C). Then they were wet oxidised at 950 °C for 90 min to yield a 380 nm thick field oxide, which passivates and protects the semiconductor surface outside the active device area.

The working electrode area was defined by patterning the field oxide by optical lithography and wet etching in BHF. The wafers were then RCA cleaned and native oxide was removed with dilute HF, and approximately a 4 nm thick tunnelling oxide was grown to the working electrode area by dry oxidation at 850 °C, in a 10% oxygen, 90% nitrogen atmosphere. A 50 nm thick Pt layer, acting as the counter electrode, was deposited by sputtering and patterned by lift-off. The oxide on the backside of the silicon wafer was wet etched and an approximately 100 nm thick aluminium layer was sputtered onto it in order to improve the backside contact.

Hydrophobic sample confinement of the device was made by depositing a hydrophobic fluoropolymer film around the electrode area by a plasma deposition process in a RIE reactor (Oxford Plasmalab 80+) using CHF_3 source gas and patterning by lift-off. Because the electrodes and contact pads were masked by photoresist during the fluoropolymer deposition process, they were left hydrophilic, whereas the surrounding areas were left strongly hydrophobic. Details of microfabrication have been published earlier [26].

2.3.2. Immunoassay

Integrated Si chips were coated with hCRP primary antibodies by physical adsorption by dispensing 57 μL coating solution on the cathode area of the chip. The coating reaction was achieved by 1 h incubation in a 100% humidity chamber. After incubation, excess coating solution was removed by suction and the coated cathode was washed by letting 4 × 1 mL of MilliQ-water flow over the electrode. The coated area was saturated to prevent nonspecific adsorption of the secondary label antibody. 90 μL of saturation solution was dispensed on the coated cathode area. After keeping

the chips in a 100% humidity chamber for 1 h, excess saturation solution was removed by suction.

After saturation, the chips were dried in room temperature for 3 h in a box with silica beads. The box with the chips was kept in a refrigerator overnight.

The immunoassay was carried out using a sandwich method, where hCRP calibration standard reacts with the coated primary antibody and then the labelled secondary antibody reacts with the hCRP standard bonded to the primary antibody.

The label used in this research was Tb(III) chelated by N1-(4-isothiocyanatobenzyl)diethylenetriamine-N1,N2,N3,N3-tetraacetate. The secondary antibody was labelled by using the reaction between the isothiocyanate group (NCS) of the Tb(III) chelate and the amino groups of the secondary antibody. 70-Fold molar excess of the chelate was used and excess label was removed later by gel filtration. The manufacturing procedure of the labelling antibody is described in detail earlier [27].

The hCRP standard was diluted into the standard dilution buffer and 5 μL of the stock solution of labelled secondary antibody was diluted to 55 μL of the label dilution buffer.

Two kinds of dispensing procedures were used in incubation. In the first one, 3.5 μL of desired standard solution was dispensed on the cathode area of the chip and straight after that, 1 μL of label solution was dispensed on the cathode area. In the second one, 5 μL of the solution containing both the antigen and labelled antibodies was dispensed on the cathode area. The same mass of antigen and labelled antibody was used in both procedures.

After 5 min incubation in the humidity chamber, each chip was washed by letting 4 × 1 mL borate–azide buffer flow over the electrode. Before the measurement, 100 μL of the borate–azide measuring buffer was dispensed on the cell area of the chip.

3. Results and discussion

In the present device, both the working and counter electrodes are integrated in the same chip, and no separate counter electrode is needed. The integrated electrodes used in this work utilise silicon wafer as the working electrode and a platinum thin film as the counter electrode.

The C/I/E tunnel junction electrodes used in HECL measurements have been, e.g. silicon oxide covered Si [1,28,29], Al, and Pt [28], aluminium oxide covered Al [12,13,28], Si, Pt and W [28], silicon nitride covered silicon [29] and magnesium oxide covered magnesium [5].

Silicon was chosen as a substrate for the chips, because it is a standard material in microfabrication and semiconductor processes and silicon wafers are readily available with high doping levels to make them electrically conductive. Thermal silicon oxide was chosen, because it is chemically robust material which works reliably and reproducibly in HECL measurements.

The chips utilise hydrophobic sample confinement, which enables dispensing straight on the surface of the cell area of the chip, because the liquid remains on the area defined by the hydrophobic ring. Therefore, no micro titer strips or separate coating vessels are needed in the assay steps. In addition, the liquid remains on the electrode during the measurement without a separately assembled sample cell.

With the current design, the volume of the reagents decreases substantially compared to the methods where micro titer strips, etc. are used. In addition, the chip set-up time is only couple of seconds which reduces the total time needed for the analysis. Cross-contamination is eliminated and no washing of any part of the measurement setup is needed between the samples.

The basic geometry of an integrated electrode HECL chip is presented in Fig. 1A, and the principle of hydrophobic confinement is shown in cross-section in Fig. 1B. The picture of the sample holder and the integrated electrode chip can be seen in Fig. 2. The size of the electrode chip is 10 mm × 19 mm.

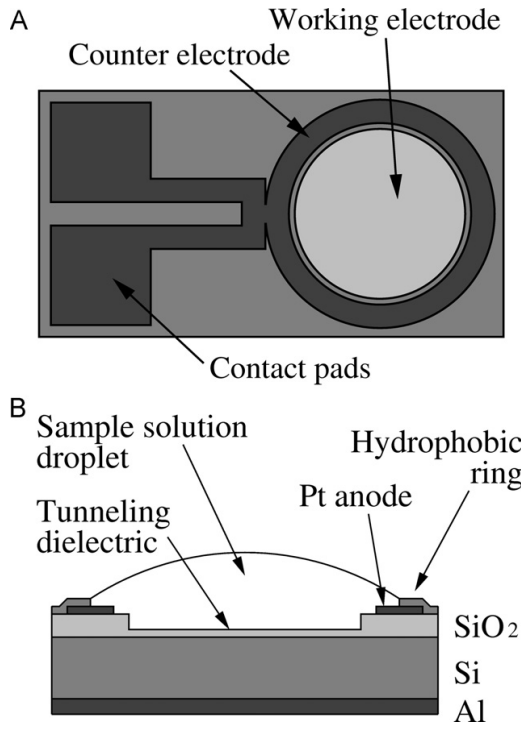

Fig. 1. (A) The geometry of the integrated electrode chip. (B) The profile of the integrated electrode chip with a hydrophobically confined sample droplet.

The Tb-chelate used in the immunoassay measurement is detectable by HECL method even at a concentration of 10^{-11} M [30,31]. This chelate has been used also earlier as a label in bioaffinity assays by HECL [31].

The chelate is excited by a ligand-sensitized mechanism where the ligand is first excited by one electron redox steps. Energy is then transferred to the Tb ion which finally emits light by $^5D_4 \rightarrow {}^7F_2$ transitions. The detailed reaction mechanism and the structure of the chelate are described earlier [30].

In this measurement the intensity was integrated over 300 excitation cycles. This integration range was selected because the

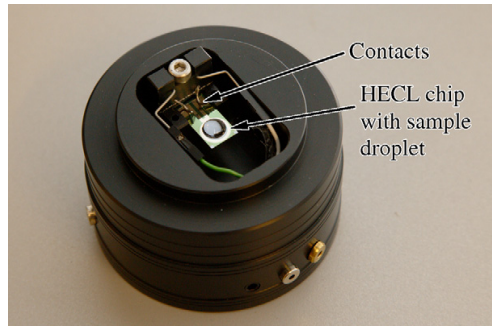

Fig. 2. The sample holder and an integrated electrode chip. The round cathode area is contacted electrically from the backside, whereas the surrounding anode is contacted by the front-side contact springs. The size of the chip is 10 mm × 19 mm.

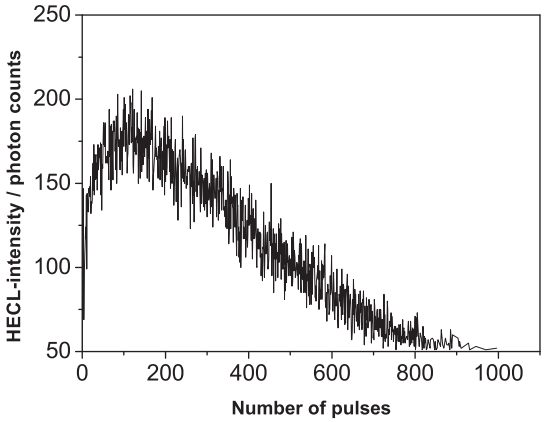

Fig. 3. HECL-intensity vs. the number of pulses in the measurement with the CRP-concentration of 10 mg L^{-1}.

intensity decreases substantially after about 300 excitation pulses, as can be seen from Fig. 3. This is not due to the detection method per se, because Tb(III) chelates can be measured over at least 1000 pulses without any decreases in the intensity [2,29]. There are several potential reasons for the intensity decrease. The most probable reason is that the protein radicals combine with the luminophore radicals and these new combinations do not luminesce. Another reason might be that the antibodies begin to break down after approximately 300 excitation pulses. The HECL intensity decreases as the breakdown products from the coating and the labels are increasing in concentration in the solution and several side reactions are able to take place due to this increase. One of the effects of the increase of the breakdown products in the analyte solution is the severe quenching of the actual luminescing compounds. However, this needs further inquiry to lead to a definite conclusion.

A calibration curve of the immunoassay is presented in Fig. 4. A CRP-immunoassay using HECL detection with integrated electrodes and hydrophobic sample confinement could be performed in the concentration range 0.01–10 mg L^{-1}. Our system

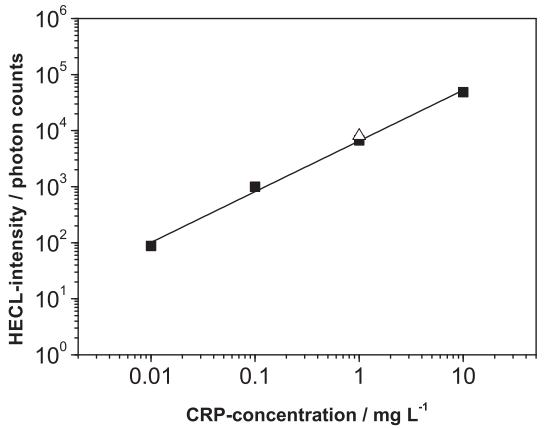

Fig. 4. Calibration curve of CRP-immunoassay by HECL-measurement using integrated electrodes with hydrophobic ring around the electrode area. The calibration curve (squares) is achieved by incubation procedure where the liquid containing both the analyte and the labelled antibody are dispensed on the electrode. The triangle describes an alternative incubation method where the analyte and the labelled antibody are dispensed separately.

thus meets the detection limit at a concentration ca. 0.01 mg L^{-1}. The concentration range is suitable for early detection biomarker studies.

The calibration curve in Fig. 4 was measured using an incubation procedure, where the liquid containing both the antigen and the labelled antibody was dispensed on the electrode. CRP concentration 1 mg L^{-1} was tested also with the other procedure, where the antigen and the labelled antibody were dispensed separately. The intensities measured with these different incubation procedures were about the same, as can be seen from the Fig. 4. According to this result it seems to be possible to get a similar calibration curve by using the other incubation procedure. When the antigen and the labelled antibody are dispensed separately, extremely low volumes, 3.5 μL and 1.0 μL, of antigen and labelled antibody are needed, respectively. This kind of procedure reduces even more the volumes of reagents needed and is especially useful when real blood samples are analysed.

It has been shown earlier that coated and saturated chips can be stored for up to one year in refrigerator and they are still usable, when coating and saturation is made on oxide covered Al electrode [31]. Similarly, coated and saturated oxide covered Si electrodes remain usable at least for one year, if they are dried at 30 °C and stored air-sealed at 2–4 °C [32]. The electrodes can be coated and saturated in advance and taken out from the storage just before use.

HECL has been utilised in immunoassays where either antigen [31] or secondary antibody [27] has been labelled with a suitable compound. In these immunoassays micro titer strips were used in assay steps and a separate counter electrode was needed during the measurement. This means higher volumes of reagents and more set-up time between the measurements than in our method using integrated electrode chip with a sample confinement.

4. Conclusions

When integrated electrodes with hydrophobic sample confinement are used, all reagents can be dispensed straight on the surface of the cathode, and separate micro titer strips, etc. are unnecessary. Besides, it makes the procedure much easier and more practical, it also reduces the reagent volumes substantially. With the present device, a separate counter electrode or a separate sample cell is not needed and therefore no washing is needed between the measurements. Fabrication of the integrated electrode devices with hydrophobic sample confinement entails only standard wafer-scale microfabrication techniques [26].

There are already some high-sensitivity CRP immunoassays available, some of them are commercialized, based on, for example, immunoturbidimetry, immunoluminometry and two-photon excitation assay technology [33–35]. However, the problem with most methods used for detection of CRP is that they are suitable only for measuring low concentrations (high-sensitivity measurements) or high concentrations (traditional measurements) [33]. It would be practical for the users if both low and high concentrations could be measured with one method. The method described in this paper is especially useful for measuring low CRP-concentrations. The wide dynamic range of the photomultiplier tube is capable of detecting CRP-concentrations up to 1000 mg L^{-1}. For such high concentrations, the amount of labelled antibody must first be optimized, but after optimization, this method should be applicable also to higher concentrations of CRP.

HECL based high-sensitivity CRP immunoassay is usable for high-sensitivity point-of-care CRP analysis because of its suitable detection range and sufficiently low detection limit. Integrated electrodes with hydrophobic sample confinement are small, disposable and very easy to use, which makes them suitable electrodes for point-of-care analysis. The present method is rapid: the

whole procedure from introduction of the antigen and labelled antibody to reading the result can be done in less than 10 min. In addition, the instrumentation is simple and easy to miniaturise.

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