Microfluidics Based System for Amperometric Determination of CRP

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Abstract

In this paper, microfluidic based system for amperometric detection of CRP is described. The amperometric sensors were designed to operate in a microfluidic system for immunoenzymatic test (ELISA). The developed microfluidic system with integrated Pt amperometric sensors is suitable for determination of CRP in the lowest concentration range valuable for clinical diagnosis - 0.1 mg/L to 1 mg/L.

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1. Introduction

A role of C-reactive protein (CRP) – non-specific marker, also considered as a predictor of cardiovascular disease, is well documented, and recommended by the European Society of Cardiology and American College of Cardiology Committee [1]. The American guideline distinguishes three ranges of the cardiovascular events risk: below 1 mg/L of CRP as a low risk, 1÷3 mg/L - a medium and above 3 mg/L as a high risk [2]. Technique commonly used for CRP determination is ELISA test. In direct ELISA tests, the first step is immunological reaction where analyte and enzyme-labeled antibodies (IgG-Enzyme) form complexes. Afterwards, product of enzymatic reaction is detected by various techniques e.g. fluorescence, spectrophotometric and others. In the case of amperometric detection, the labeling enzyme is selected in such a way that product of the reaction catalyzed by the enzyme is an electrochemically active substance. Then, the product of enzymatic reaction is electrochemically...
oxidized/reduced at the working electrode (WE). The redox current generated in this reaction – a measurand, is proportional to concentration of analyte-IgG-Enzyme complexes.

In our experiments, as a label for anti-CRP antibodies alkaline phosphatase (AP) which catalyses hydrolysis of ascorbic acid phosphate (AAP) was used [3]. Then, the electroactive product of the enzymatic reaction – ascorbic acid (AA) – undergoes oxidation to dehydroascorbic acid (DAA) at the electrode surface (Fig. 1).

Fig. 1. Principle of immunosensor operation, where: AAP - ascorbic acid monophosphate, AA - ascorbic acid, DAA - dehydroascorbic acid.

2. Experimental

Two types of amperometric sensors with: Pt thin-film electrodes fabricated by magnetron sputtering on Si substrate and lift-off technique as well as thick-film - carbon and graphite working electrodes made using microdosing robot Ultra TT (EDF) were developed, in particular:

- thick-films based on Autostat CT 175 μm with:
  - carbon working electrode (WE) of surface area S = 4.5 mm², carbon counter electrode (CE), and Ag/AgCl as a reference electrode (RE),
  - graphite WE of surface area S = 4.5 mm², graphite CE, and Ag/AgCl as a RE,
- thin-film platinum WE (surface area - 1.4 mm²) and platinum CE, Ag/AgCl wire as an external RE.

In the first stage of this study, different materials of working electrode were investigated. In experiments, a typical for the AP-based enzymatic reaction buffer solution i.e. 0.01 M Tris of pH 9 containing 0.1 M KCl and 1 mM MgCl₂ was used. First, responses of the amperometric sensors in solutions containing free AP-labeled antibodies (IgG-AP) in concentration range 0 ÷ 1 mg/L were examined. The responses were recorded after 40-minute incubation of IgG-AP with 700 μL of 10 mM AAP at room temperature, using cyclic voltammetry (CV) method – on VMP2/Z (PAR, USA) potentiostat, with scan rate 50 mV/s and in potential range of –0.2 ÷ +1.0 V.

Next, the graphite and platinum sensors were examined for detection of human CRP (0 ÷ 3 mg/L), applying immersion method based on direct ELISA test utilizing anti-CRP IgG (Genetex). The antibodies were labeled with AP using alkaline phosphatase labeling kit (Kit-NH₂, Abnova). The 1.5 cm² nitrocellulose membranes (HAHY, Immobilon) were coated with 100 μL of given CRP solution (in PBS with Tween 20) and incubated at 37°C for 1 hour. After washing step (PBS with Tween 20), blocking of the membranes with 5% BSA in PBS buffer was carried out at 4°C overnight and then, the back side of membranes was blocked in the same solution at 37°C for 15 minutes. After next washing step, the membranes were coated with 70 μL of AP-labeled IgG solution and incubated at 37°C for 1 hour. Then, the membranes were washed and incubated in 700 μL of 10 mM AAP (in Tris pH 9) at room temperature for 40 minutes.

The developed microfluidic system consisted of two parts: glass plate (Pyrex, Schott) with sputtered platinum electrodes and polydimetoxysiloxane - PDMS structure with microchannels (Fig. 2.a). Detection chambers of 1.2 μL or 2.4 μL volume contained the thin-film Pt/Ti (300 nm/10 nm). Active surface of
WE was 1 mm². The measurements were performed vs. external calomel saturated electrode (REF 401, Radiometer), using potentiostat/galvanostat PalmSens (Palm Instruments BV, The Netherlands). Fluid flow was run by peristaltic pump (Ismatec REGLO Analog/Digital) equipped with Tygon tubing (Fig. 2.b).

For AA calibration, chronoamperometric measurements were performed in 1.2 μL chamber at oxidation potential +0.8 V and at different flow rates from 100 to 400 μL/min. The AA standard in Tris buffer (pH 9) was added to change the concentration up to 13.5 mM in 3-minute time intervals. For the CRP detection - using the same procedure of direct ELISA test as described above, CV curves were recorded in 2.4 μL detection chamber at potential range of −0.2 ÷ +1.0 V with scan rate 50 mV/s.

3. Results and discussion

To compare performances of the amperometric sensors and eliminate differences in surface area of working electrodes, the current density was used as a mesurand. After 40-minute incubation of free IgG-AP with AAP, average response of the sensors reached about 70% of the final response - i.e. response after 60-minute incubation. Comparing the sensors with different working electrodes: carbon, graphite and platinum, it can be concluded that the highest current density was obtained for the sensors with graphite working electrode (Fig. 3.b). For the sensors, the obtained IgG-AP lower detection limit was 0.0625 mg/L.

![Fig. 2. (a) View of the fragment of microfluidic system with Pt electrodes; (b) Schematic illustration of system for amperometric measurement in microfluidic chip.](image)

![Fig. 3. Dependence between IgG-AP concentration and oxidation current density for different electrode materials: graphite (G), platinum (Pt) and carbon (C).](image)
The chronoamperometric response of Pt sensors in the microfluidic system to AA concentration range up to 13.5 mM was obtained with very good reproducibility (Fig. 4.a). In the microfluidic system as well as in immersion mode, the amperometric sensors were investigated for the human CRP concentration range of 0 ÷ 3 mg/L. Exemplary dependence between CRP concentration and current density calculated for the sensor with graphite working electrode used in immersion mode and the Pt sensor used in microfluidic system is shown Fig. 4.b.

Fig. 4. (a) Chronoamperometric response of the Pt electrode (potential +0.8 V) operating in a microfluidic channel to changes of AA concentration up to 13.5 mM for different flow rates: 100, 200 and 400 μL/min; additions done at 3-minute time intervals, (b) Dependence between CRP concentration and oxidation current density for different sensors: graphite WE in immersion method (G), platinum WE in the microfluidic system (Pt)

4. Conclusions

A microfluidic system with integrated amperometric sensors for CRP determination based on immunoenzymatic test, namely enzyme-linked immunosorbent assay (ELISA) was developed. The microsystem with Pt amperometric sensors is suitable for determination of CRP in the lowest concentration range valuable for clinical diagnosis i.e. 0.1 mg/L to 1 mg/L.

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