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# Occlusion phenomenon of redox probe by protein as a way of voltammetric detection of non-electroactive C-reactive protein

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ABSTRACT

Simple, selective and sensitive analytical devices are of a great importance for medical application. Herein, we developed highly selective immunosensor for electrochemical detection of C-reactive protein (CRP) in blood sample. Branched polyethylenimine functionalized with ferrocene residues (PEI-Fc) was the main element of the recognition layer, which allowed: (i) covalent binding of an antibody in its most favorable orientation and (ii) voltammetric detection of the C-reactive protein. Anchoring of PEI-Fc to the electrode surface through the electrodeposition process leads to the formation of thin, stable and reproducible layers, which is extremely important in the case of electrochemical immunosensing. The proposed analytical device is characterized by high selectivity and sensitivity and can be successfully used in the concentration range of CRP from 1 to $5{\cdot}10^4$ ng $mL^{-1}$. The determined limit of detection was *circa* 0.5 and 2.5 ng $mL^{-1}$ for voltammetric and impedance analysis, respectively. The developed analytical device has also been successfully applied for the analysis of CRP level in rat blood samples.

## 1. Introduction

C-reactive protein (CRP) is the acute-phase protein which is a well-known diagnostic biomarker of different inflammatory processes and tissue damages in humans (Gabay and Kusher, 1999; Young et al., 1991). In general, plasma CRP is produced mainly by hepatocytes, predominantly under transcriptional control by the cytokine IL-6, although other sites of local CRP synthesis and possibly secretion have been also discovered (Agassandian et al., 2014; Anty et al., 2006; Brooks et al., 2010; Hurlimann et al., 1996; Jabs et al., 2003). In healthy humans, plasma CRP is averaged for 3–10 mg $L^{-1}$, but following acute-phase triggers, values may significantly increase to more than 500 mg $L^{-1}$ (Vigushin et al., 1993). In contrast, plasma CRP due to acute-phase stimulus may elevate only up to 30 mg $L^{-1}$ in mice, but is raised to even more than 700–900 mg $L^{-1}$ in both rats and rabbits (de Beer et al., 1982; Torzewski et al., 2014). Note the major CRP acute-phase responses correlate closely with different infection stages and cirrhosis (Pieri et al., 2014), allergic complications of infection due to rheumatic fever or erythema nodosum (Gölbasi et al., 2002), inflammatory diseases such as juvenile chronic arthritis (Gwyther et al., 1982), ankylosing spondylitis (Benhamou et al., 2010), psoriatic arthritis and systemic vasculitis (Jain and La, 2011), necrosis states due to myocardial infarction or atherothrombotic events (Calabrò et al., 2012), trauma (Baumeister et al., 2016) and some malignancies including lymphomas, carcinomas and sarcomas (Allin and Nordestgaard, 2011). Therefore, routine laboratory use of CRP measurements is widely applied in clinical practices due to its usefulness in screening tests for some organic diseases, assessment of disease activity in inflammatory conditions, diagnosis and managements of infection and classification of inflammatory diseases and cancers (Bassuk et al., 2004). Since even discrete circulating CRP values may correlate well with a number of different pathologies in humans, predictive associations of plasma CRP with such events is really needed to be assayed due to highly sensitive analytical approaches.

In clinical laboratories, a number of original methods for CRP detection like turbidimetry, nephelometry and ELISA standard enzyme immunoassay are used (Komoriya et al., 2012; Roberts et al., 2001). However these methods are time-consuming, expensive, prone to false negatives, no sensitive (limit of detection *ca.* 0.1 mg $L^{-1}$), require skillful operation, qualified personnel and sophisticated

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instrumentation. Therefore a rapid, selective, ultrasensitive protocols for determination of trace CRP levels are still needed. The electrochemical methods are the most promising in the terms of low cost, low sample volume, high sensitivity, wide dynamic concentration response range and versatility (Jampasa et al., 2018; Kokkinos et al., 2015; Wang et al., 2017). The electrochemical protocols are mainly based on the antibody-antigen specific interactions or on the aptamer-protein interactions (Rahi et al., 2016; Wang et al., 2017). To enhance the sensitivity of such CRP immunosensors various nanomaterials including metal nanoparticles as Au, Hg and Bi (Kokkinos et al., 2015), quantum dots (Bing and Wang, 2017), poly(dimethylsiloxane)-gold nanoparticle composite (Zhou et al., 2010), vertically aligned carbon nanofibers with generated carboxylic acid groups (Gupta et al., 2014), composite of polyaniline with molybdenum disulfide (Zhang et al., 2016) have been applied as carriers for immobilization of biomolecules or signal molecules. Nevertheless, the application of nanomaterials do not meet all analytical requirements due to multiple preparation steps, toxicity of heavy metal nanoparticles and not enough sensitivity. There are still many points to upgrade, such as simplification of the biosensor construction, application of non-toxic elements, design of new high sensitive redox labels for electrochemical detection of CRP.

To construct a simple, low-cost, portable and high selective and sensitive electrochemical immunosensor for voltammetric determination of C-reactive protein in blood samples the branched polyethylenimine modified with ferrocene (PEI-Fc) was used as a platform for covalent anchoring of monoclonal antibodies directed against CRP. PEIs have gained enormous attention in novel drug delivery systems (Neuberg and Kichler, 2014) and modern electrochemistry (Kahlouche et al., 2018). The detection was based on the occlusion phenomenon of Fc residues resulting on the diminishing of its voltammetric signal. The analytical characteristic of the proposed device was accomplished by using various techniques.

## 2. Experimental section

The details about used chemicals, procedure of the synthesis of ferrocene-functionalized polyethylenimine (PEI-Fc) and applied techniques are given in Supporting Information, Section 1.

### 2.1. Preparation of CRP immunosensor

The electrode surface was electrochemically modified with PEI (0.8 kDa)-Fc film during two cyclic voltammograms recorded in the potential range 0–0.7–0 V, with scan rate 100 mV $s^{-1}$, in 1 mg $mL^{-1}$ solution of PEI-Fc (in 0.1 M phosphate buffer (PB) with addition 0.15 M $K_2SO_4$; pH 7.0). After electrodeposition, the polymeric film was stabilized by recording continuous cyclic voltammograms in buffer until a stable voltammogram was observed. The presence of free amino groups in the PEI-Fc structure allowed the covalent conjugation of an antibody to a recognition layer. After the activation step (Hermanson, 2013), the 20-μL droplet of 50 μg $mL^{-1}$ antibody solution in 0.1 M PB with addition 0.15 M $K_2SO_4$ was placed onto the electrode surface and kept under the cover at 4 °C for 2 h. Next, the unbounded antibody molecules (Ab) were removed by careful rinsing with ultrapure water. After this step the sensor was incubated with BSA blocker to bind nonspecific binding sites to prevent nonspecific adsorption (1 mg $mL^{-1}$ BSA in 0.1 M PB with 0.15 M $K_2SO_4$). Then the 20-μL droplet of buffer solution containing the appropriate concentration of C-reactive protein was uniformly deposited on the recognition layer surface and allowed to react at 4 °C for 2 h under the cover. After that time the surface was carefully rinsed with ultrapure water and differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) measurements were performed. The whole procedure of the construction of the developed immunosensor for CRP detection is presented in Scheme 1.

## 3. Results and discussion

### 3.1. Characteristic of recognition platform

Generally, the absolute value of primary amino groups of PEI is not known. The relative percentage of amino groups in PEI can be evaluated, only. The presence of free amine groups in PEI was confirmed by inverse-gated $^{13}C$ NMR studies, see Fig. 3S in Supporting Information. Inverse-gated 13C NMR is a commonly applied technique for evaluation of the branched polymer modification level (Cao et al., 2008; Krämer et al., 2004; Li et al., 2012). Therefore, the inverse-gated $^{13}C$ NMR provided the reliable information on the relative content of Fc moieties in the polymeric material. The calculated $NH_2$:NH:N ratio for PEI-Fc equals 1.00:4.51:2.40. The ratio $NH_2$:NH:N for the modified PEI is different than for the pristine polymer (1.00:1.12:0.89) (Kasprzak et al., 2016), which further supports our thesis on the covalent modification of PEI using Fc-CHO. Modification level of primary amines of PEI in PEI-Fc material equals *ca.* 20%. This number also stands for a relative content of Fc in the polymeric material. It is noteworthy that the applied molar amount of formyl groups coming from ferrocenecarboxaldehyde (Fc-CHO) with the respect to primary amino groups of PEI resulted in the satisfactory modification level of the polymer and provided appropriate control on the addition process (Kasprzak et al., 2015, 2016, 2018).

To optimize the thickness of the polymer layer different number of voltammetric cycles during deposition process were applied. As it is seen in Fig. 1A, the intensity of the oxidation peak current initially increased with increase of the number of cycles applied during deposition step and reached a maximal value after 2–3 CV cycles. For more than 3 number of cycles the current value started to slightly decrease. With increasing number of cycles the separation of voltammetric peaks ($\Delta E$) also increased (Fig. 1A). It means that the electron exchange was hindered. Hence, the number of deposition cycles was chosen as 2. Detailed electrochemical characteristic of PEI-Fc layer is presented in Supporting Information, Section 2.1.1.

The formation of subsequent layers during each electrode modification step was monitored by using electrochemical impedance spectroscopy and quartz crystal microbalance with dissipation. The obtained Nyquist plots presented in Fig. 1B and C were characterized by a semicircle followed by a straight line at 45°, which is typical for diffusion-controlled processes. The modification of GC electrode by the electrodeposition of PEI-Fc results in a significant decrease in the charge transfer resistance ($R_{ct}$, diameter of the semicircle). This may indicate that the amino groups in the polymer network are protonated and form positively charged film at the electrode surface. In such case the electrostatic attraction between the negatively charged redox probe present in the solution ($Fe(CN)_6^{4-/3-}$) and positively charged polymer film took place. The covalent anchoring of the antibody molecules to a polymer network and then the introduction of CRP at the electrode surface, leads to an increase in the $R_{ct}$. It indicates that the electron exchange between redox probe and the electrode surface is less effective. The greater increase of $R_{ct}$ value was observed after the interaction of the recognition layer (GC/PEI-Fc/Ab) with C-reactive protein. This effect can be explained by the appearance of the additional layer at the electrode surface, which hindered the electron exchange.

It is known that the presence of antibody in the recognition layer provides high selectivity of the sensor. However, the orientation of Ab molecules in the layer is a crucial step to achieve a full access of antigen to the antigen binding site located in the Fab (fragment, antigen-binding) region of Ab. Depending on the way of introduction of Ab molecules into the recognition layer and the concentration of Ab in solution a following Ab orientations can be achieved: flat (horizontal), tilted (side-on) and vertical (Stobiecka et al., 2016). In our case the antibody was covalently anchored to a polymer layer *via* the amide-type linkage between amine groups of PEI and carboxyl groups on the surface of Fc (fragment, crystallizable) region of Ab. Moreover, the high