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Detection of biomarkers for inflammatory diseases by an electrochemical immunoassay: The case of neopterin

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ABSTRACT

An electrochemical immunoassay for neopterin was developed using recently produced specific antibodies immobilized to protein A-coated magnetic beads in combination with differential pulse voltammetry and screen-printed array of electrodes. Neopterin–alkaline phosphatase conjugate was used as label in a competitive assay format. Multiplexed analysis of neopterin was demonstrated by replacing the traditional ELISA with electrochemical detection and the traditional plastic wells with screen-printed array of electrodes. The optimized electrochemical method, based on polyclonal antibodies, reached a limit of detection of 0.008 ng/mL with an average RSD %= 10. Serum samples collected from patients with sepsis, healthy volunteers and other patients without a confirmed clinical diagnosis were also analyzed. The obtained results, compared with those of a commercial ELISA kit, had a significant correlation, showing the possibility to distinguish among the serum samples from ill or healthy subjects.

1. Introduction

Neopterin, a pteridin of low molecular mass (253 g/mol), is a catabolic product of guanosine triphosphate. Increased neopterin concentrations in body-fluids, such as serum or urine, indicate cellular immunity activation and have been observed in diseases like viral infections, including HIV infection and infections by intracellular living bacteria or parasites, inflammatory diseases, autoimmune diseases, neurodegenerative diseases, certain types of cancer and many other pathologies [1–6]. Neopterin measurements not only allow evaluating the extent of cellular immune activation but also the extent of oxidative stress and increased production of reactive oxygen species (ROS) [3,7]. The relationship between neopterin and risk of heart failure has yet to be studied on a large scale, but a correlation between neopterin, as a marker of monocyte activation, and the risk of hospitalization for heart failure has been highlighted [8]. Recent studies have demonstrated an association between increased neopterin levels and future risk of recurrent acute coronary syndrome events, suggesting serum neopterin as probably the best single predictor of death in healthy individuals with angiographic abnormalities [9–11].

Regarding the use of neopterin as a valuable biochemical marker of cellular immunity [1,12], the average normal concentration of serum neopterin in healthy adults was reported to be less than 2.23–2.46 ng/mL [13] and a cut-off value for diagnostic ELISA tests was set to be 3.04 ng/mL [6]. Neopterin concentrations higher than the cut-off value were considered to be elevated levels, signalizing the activation of the human immune system.

Several strategies have been developed for the detection of neopterin in biological samples, such as capillary electrophoresis, high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), fluoroimmunoassay and enzyme-linked immunosorbent assay (ELISA) [14–17].

However, with the exception of ELISA test, these methods have many disadvantages such as employing expensive labeling detection methods, are time consuming and require qualified personnel and sophisticated instrumentation. Alternative methods are urgently desirable.

Electrochemical biosensors appear as promising tools for point-of-care testing due to low cost, ease of miniaturization, and possibility of integration with multi-array tools.

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With the aim to increase the sensitivity of the assay, as well as to improve the performance of the immunological reaction and the speed of its kinetics, recently the development of functional monoclonal and polyclonal antibodies with high degree of specificity and affinity towards neopterin has been reported in literature [18]. The produced antibodies and hapten conjugates were analytically characterized in conventional ELISA formats and their detection capabilities were verified in clinical samples. The produced monoclonal antibodies reached in direct ELISA format a limit of detection (LOD) of 0.18 ng/mL. The polyclonal antibodies were somewhat more sensitive in direct ELISA with LOD of 0.05 ng/mL [18]. In recent times, the same monoclonal antibodies were also tested in a binding inhibition assay based on fluorescence measurement and a LOD of 0.45 ng/mL was reported [19,20].

In this work, we report for the first time an electrochemical immunosensor for the detection of neopterin. To the best of our knowledge, there are no reported electrochemical immunosensors for the detection of neopterin, though different biosensors have been developed for the detection of other markers of inflammation, sepsis and cardiac pathological status [21–24].

Herein, polyclonal and monoclonal antibodies [18] were used to develop an electrochemical bioassay, coupling magnetic beads with screen-printed array of electrodes [21,25,26]. Protein A-coated magnetic beads were modified with the capture antibody. Neopterin–alkaline phosphatase conjugate was used as label in a competitive assay scheme. This because, neopterin is too small for providing two epitopes; therefore, a competitive format was used rather than a sandwich immunoassay. Serum samples collected from patients with sepsis, healthy volunteers and other patients without a confirmed clinical diagnosis were also analyzed.

2. Experimental

2.1. Chemical reagents and immunoreagents

All the reagents for the buffers were from Merck (Milan, Italy). Neopterin, bovine serum albumin (BSA), α-naphthyl phosphate (NP) and human serum were provided by Sigma-Aldrich (Milan, Italy). Due to poor solubility in water, neopterin stock solution was prepared in 1 M hydrochloric acid and stored at 4 °C protected from light. Protein A-coated magnetic beads (Dynabeads® protein A) were purchased from Dynal Biotech (Milan, Italy). Monoclonal (mAb) and polyclonal antibodies (pAb) against neopterin and the neopterin–alkaline phosphatase conjugate (neopterin–AP) used in this study were provided by Prof. M. Franek, Veterinary Research Institute, Brno, Czech Republic. Neopterin ELISA kit was purchased from DRG International Inc. (Mountainside, NJ, USA). The buffers used for the experiments are the following:

- solution A for washing and immobilization of antibodies: 0.1 M sodium phosphate solution pH 8;
- buffer B (working assay buffer) for competition: 100 mM sodium phosphate buffer pH 7.4 containing 100 mM NaCl, BSA (5 g/L) and 0.005% (v/v) of Tween 20;
- buffer C (detection buffer): 0.1 M diethanolamine buffer containing 1 mM $MgCl_2$ and 100 mM KCl pH 9.6.

2.2. Electrochemical instrumentation

Electrochemical measurements were performed using μAutolab type II PGSTAT with a GPES 4.9 software package (Metrohm, Italy). All the measurements were carried out at room temperature by using differential pulse voltammetry (DPV) with the following parameters: potential range 0/+600 mV, step potential 7 mV, modulation amplitude 70 mV, standby potential +200 mV, interval time 0.1 s.

The transducers were screen-printed eight-electrode arrays based on eight graphite working electrodes (diameter=2 mm), each with its own silver pseudo-reference electrode and graphite counter electrode [27]. The arrays were screen-printed in-house using a DEK 248 screen-printing machine (DEK, Weymouth, UK). Silver based (Electrodag PF-410) and graphite-based (Electrodag 423 SS) polymeric inks were obtained from Acheson (Milan, Italy); the insulating ink (Vinylfast 36–100) was purchased from Argon (Lodi, Italy). A polyester flexible film (Autostat CT5), obtained from Autotype (Milan, Italy), was used as printing substrate [28].

The printed arrays are cut in strips of dimension 40 mm × 84 mm. 24 contact pins allow connecting them to the electrochemical instrument. An 8-holes methacrylate box (4 mm × 84 mm × 5 mm) is fixed onto the strip by using a double layer adhesive. Each hole is 8 mm in diameter and it is positioned exactly in correspondence of each sensor of the array, allowing producing an 8-cells electrochemical array. Each array is finally placed in a holding block with eight magnet bars with a diameter of 1.5 mm. The sample mixer with a 12-tube mixing wheel and the magnetic separator with 6-tube positions were purchased from Dynal Biotech (Milan, Italy).

2.3. Electrochemical competitive assay

A competitive assay was carried out using protein A-coated magnetic beads as solid support for the immunoassay and carbon screen-printed arrays as transducers. A scheme of the electrochemical competitive assay is shown in Fig. 1.

The competition curves were analyzed with a four-parameter logistic equation using a proper software (Graph Pad, Prism 4 for Windows, Graph Pad Software Inc.) according to the following formula:

$$Y = A + \frac{(B-A)}{1+10^{[\log\ EC_{50} - X]^{Hillslope}}}$$

where A is the Y-value at the bottom plateau of the curve, B is the Y-value at the top plateau of the curve, EC_{50} is the antigen concentration necessary to have the 50% of the signal and Hillslope is the slope of the linear part of the curve.

2.3.1. Immobilization of antibodies on magnetic beads

Polyclonal and monoclonal antibodies against neopterin were immobilized onto protein A-coated magnetic particles according to the manufacturer's instructions. This kind of particles allow performing an oriented immobilization of antibody molecules, considering that protein A selectively binds the Fc domain of antibodies.

At this purpose, magnetic beads were first washed with 0.1 M sodium phosphate solution pH 8 to remove the NaN_3 preservative, then a suspension of 100 μL was introduced in a tube containing 500 μL of anti-neopterin IgG (100 μg/mL) prepared in solution A. After 20 min of incubation, the tube was positioned on a magnet holding block, the supernatant was removed and beads were washed twice with 500 μL of solution A. Each washing step consisted in a re-suspension of the beads in the washing solution for 2 min, followed by the separation with the magnetic holding block to remove the supernatant. In this way, antibody-coated beads were obtained. Antibody-coated particles could be prepared in advance and stored at 4 °C for many weeks.

2.3.2. Affinity reaction and electrochemical measurement

To carry out the competitive assay, the following solutions were incubated for 20 min: