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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:

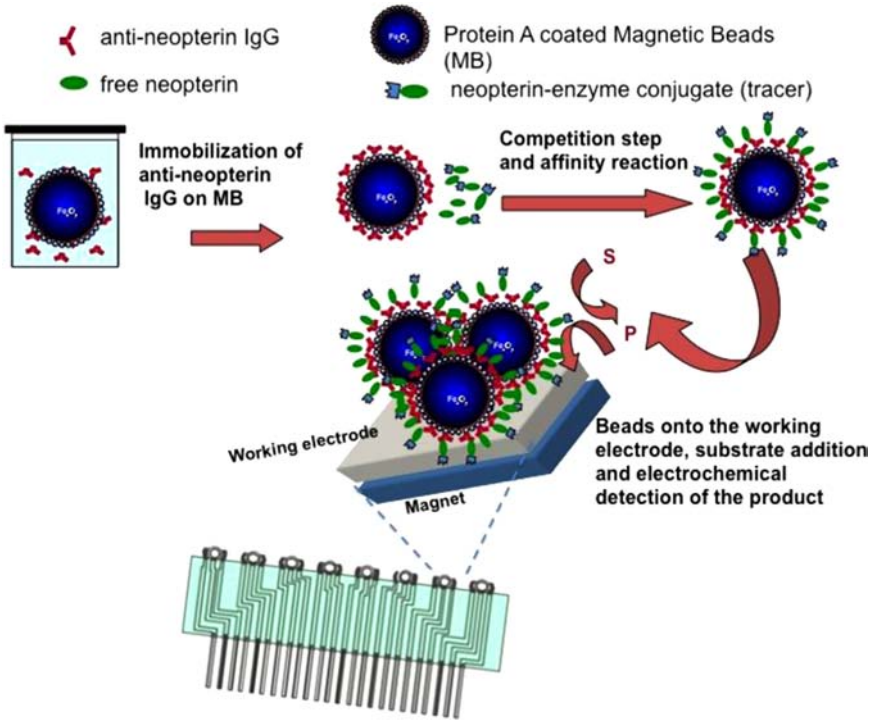

Fig. 1. Scheme of the electrochemical competitive assay.

- 350 μL of buffer B;
- 50 μL of neopterin–AP solution at a dilution of 1:100 (final dilution 1:1000);
- 50 μL of antibody-coated beads suspension;
- 50 μL of neopterin standard or sample.

After incubation, the beads were magnetically separated and the supernatant was removed. After two washing steps, the beads were re-suspended in 100 μL of buffer B and 10 μL of the suspension was transferred onto the surface of each working electrode of the array (by keeping them in the correct position with the aid of the magnet holding block). Then 60 μL of a 1 mg/mL solution containing the enzymatic substrate (α-naphthyl phosphate) prepared in buffer C was deposited onto each well, making attention to close each electrochemical cell. After 5 min, the enzymatic product was determined by DPV. The height of the obtained peak was used as analytical parameter.

2.3.3. Calibration curve in commercial serum and clinical samples

Standard solutions of neopterin were added to commercially available human serum diluted 1:10 to test the performance of the assay in a complex matrix. Moreover, serum samples from 13 subjects were analyzed. All samples were taken in a sterile tube for serum observing the usual precautions for venipuncture and keeping away from heat and direct sun light. Serum samples, stored at −20 °C, were tested by the electrochemical competitive assay, using polyclonal antibodies and a commercial ELISA Kit. The results of the two methods were compared in terms of B_x/B_0 (signal of the samples/signal of the blank). All experiments were performed in compliance with the relevant laws and institutional guidelines, and the experimentation with human samples was approved by the Ethical Committee of AOUP of Pisa, Italy (Study 2271/07; Protocol CARE-03 WP10).

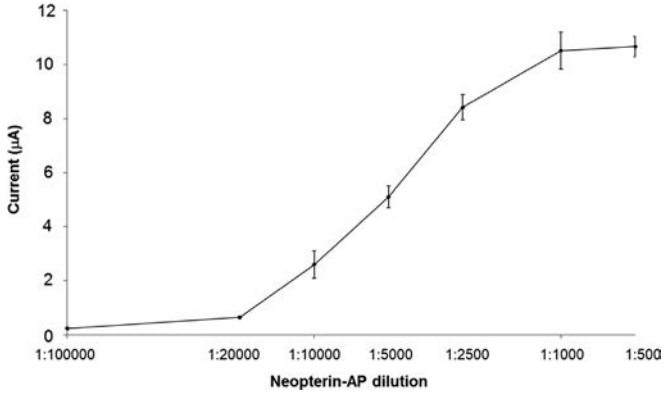

Fig. 2. Choice of neopterin–AP dilution to use for the competitive assay. The immobilization time of the monoclonal antibody solution (100 μg/mL) on protein A-coated beads and that of incubation between antibody-coated beads and the neopterin–AP solution is 20 min. The points correspond to the average current ± S. D. calculated for $n=4$ repetitions. The dilution 1:1000 is chosen to perform the competition.

3. Results and discussion

3.1. Competitive assay with mAb

In order to find the best conditions for the competitive assay based on the use of monoclonal anti-neopterin antibodies, the dilution of neopterin–AP conjugate solution and competition time were optimized. An antibody concentration of 100 μg/mL and an immobilization time of 20 min were used. Different dilutions of neopterin–AP, in the dilution range 1:500–1:100,000, were tested. The results are presented in Fig. 2, in which the typical behavior of a binding curve is shown. The current values increased when the tracer concentration increased and for dilutions 1:500 and 1:1000 the currents reached a steady state indicating that all antibodies sites onto the magnetic beads were saturated. The dilution 1:1000 was chosen to perform the competition.

Moreover, the competition time, i.e. the time necessary to the affinity reaction to reach completion, was optimized. These experiments were performed by incubating the antibody-coated magnetic beads with the competition solution containing a fixed dilution of the neopterin–AP conjugate (1:1000 with respect to the stock solution) and neopterin (40 μg/L) at different times. These signals were compared with those obtained for the same experiment in absence of free neopterin (100% of signal). The comparison between the recorded signal in presence and in absence of neopterin is presented in Fig. 3; 10 min of incubation was chosen, as competition time, due to the best discrimination between the signal without and with neopterin.

A dose–response curve of neopterin was carried out using the above-optimized conditions (Fig. 4). The signal is reported as B_x/B_0 percentage units, that is the percentage of the signal decrease with respect to the blank value.

The height of the peaks obtained by DPV measurements for different concentrations of neopterin decreased with the increase of neopterin concentration showing a typical behavior of a competitive assay.

In order to evaluate the reproducibility, four repetitions of each standard solution in the concentration range 0–400 ng/mL were carried out. The average CV was 8%, calculated as the mean of all the concentrations considered. The LOD of the assay was calculated by the evaluation of the average response of the blank minus three times the standard deviation. In this case, an EC_{50} of 2.1 ng/mL and a LOD of 0.5 ng/mL were achieved, which both were included in the physiological concentration range of neopterin.

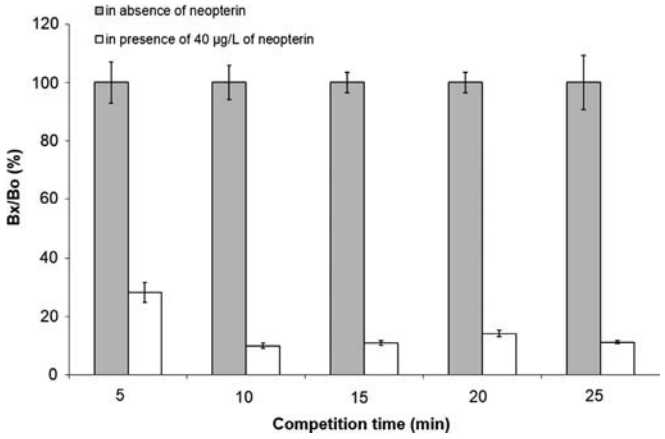

Fig. 3. Optimization of the competition time. The immobilization time of the monoclonal antibody solution (100 μg/mL) on protein A-coated beads is 20 min and the dilution of neopterin–AP conjugate is 1:1000. The points correspond to the average current ± S.D. calculated for $n=4$ repetitions.

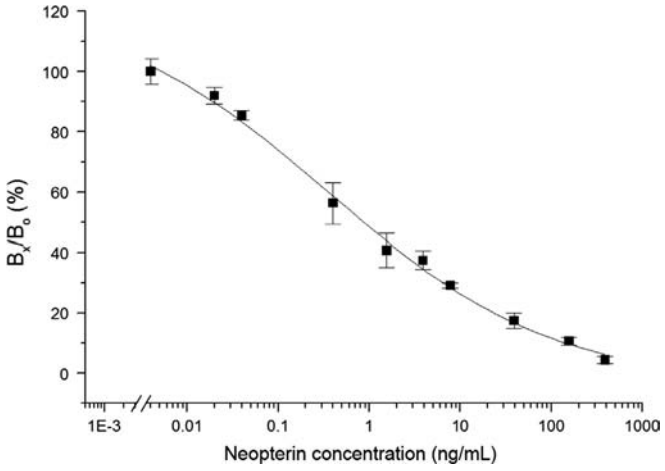

Fig. 4. Neopterin dose–response curve. Competitive assay conditions: monoclonal antibody concentration: 100 μg/mL, immobilization time: 20 min, neopterin–AP dilution: 1:1000, competition time: 10 min. The points correspond to the average current ± S.D. calculated for $n=4$ repetitions.

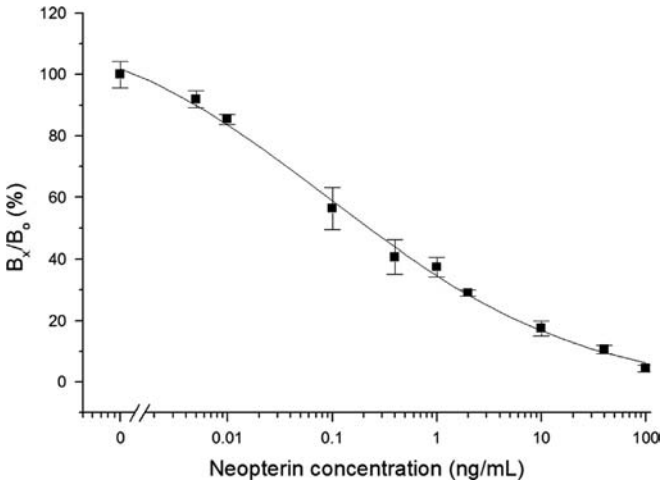

Fig. 5. Neopterin dose–response curve. Competitive assay conditions: polyclonal antibody concentration: 100 μg/mL, immobilization time: 20 min, neopterin–AP dilution: 1:1000, competition time: 20 min. The points correspond to the average current ± S.D. calculated for $n=4$ repetitions.

3.2. Competitive assay with pAb

The same parameters (neopterin–AP dilution and competition time) studied in the case of the monoclonal antibody were optimized also for the development of the competitive assay using the polyclonal antibody. A dilution of 1:1000 of the enzymatic conjugate and a competition time of 20 min resulted the best conditions for the competitive assay (data not shown). A dose–response curve of neopterin was performed using such parameters (Fig. 5). In this case, better results were achieved with EC_{50} and LOD values of 0.09 ng/mL and 0.008 ng/mL, respectively. In order to evaluate the reproducibility, four repetitions of each standard solution in the concentration range 0–100 ng/mL were carried out. The average CV was 10%, calculated as the mean of all the concentrations considered. The obtained LOD was even better of that reported for the colorimetric ELISA [18].

3.3. Application of the electrochemical competitive assay to spiked commercial serum samples and clinical samples

Finally, the ability of the electrochemical competitive assay to detect neopterin in a complex biological matrix such as serum was evaluated. Polyclonal antibody-coated beads were used for these experiments due to the higher sensitivity obtained with the standard solutions. The following optimized parameters were used:

(a) *Commercial Serum:* Serum, diluted 10 times with buffer B, was spiked with neopterin (in the concentration range 0–85 ng/mL), and the results were compared with those obtained in buffer. Fig. 6 shows that comparable responses were found for both buffer and spiked serum samples: addition of neopterin to the sample resulted in protein concentration-dependent signal. A weak matrix effect was observed considering the higher currents measured in serum with respect to buffer.

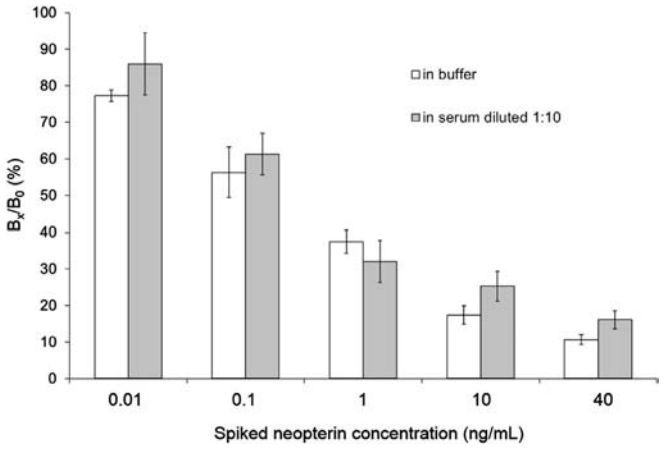

Fig. 6. Results obtained with serum samples spiked with neopterin (gray histograms) and comparison with the same concentrations tested in buffer (white histograms).

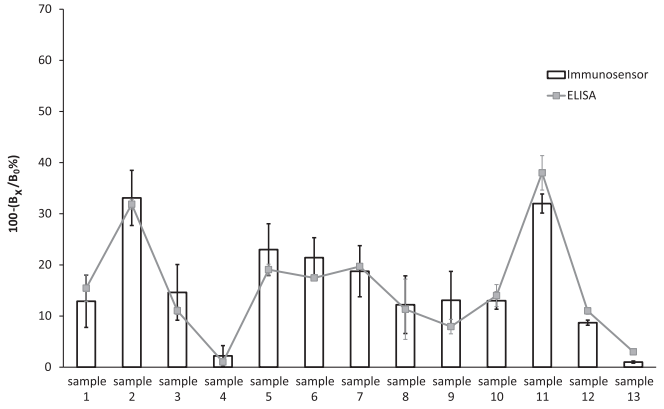

Fig. 7. Real samples analysis: $(100-B_x/B_0)$ for the electrochemical immunoassay (bars) and the commercial ELISA test (gray squares). Competitive assay conditions: polyclonal antibody concentration: 100 μg/mL, immobilization time: 20 min, neopterin–AP dilution: 1:1000, competition time: 20 min. The points correspond to the average current ± S.D. calculated for $n=3$ repetitions.

(b) *Clinical samples:* The measurement within a spiked serum sample is a good tool to characterize the system under realistic conditions of a real matrix. Nevertheless, it is just a simulation. The measurement of serum samples obtained from healthy patients and patients with clinical parameters is a challenge, which has to be taken in consideration in developing new methods. Thus, 13 serum samples were tested with the electrochemical immunoassay. The final concentration of neopterin in all samples was determined by a commercial ELISA test as reference technique. The results of the two methods were compared in terms of B_x/B_0 (signal of the sample/signal of the blank), as shown in Fig. 7. Both the methods have a similar behavior. As confirmed by the ELISA analysis, samples 4 and 13 (neopterin concentration 1.30 ± 0.02 ng/mL and 1.50 ± 0.003 ng/mL, respectively) were from healthy volunteers, whereas samples 2 and 11 (neopterin concentration 4.9 ± 0.2 ng/mL and 6.5 ± 0.2 ng/mL, respectively) were from patients with sepsis and the other samples were from subjects without a confirmed clinical diagnosis. B_x/B_0 signals from clinical samples were highly consistent with results obtained with ELISA test. The experimental correlation coefficient value obtained (0.950) was compared with the 5% significance value for Pearson's r (0.521; n (degree of freedom)=11) in one tailed Pearson's test [29], confirming a significant correlation among commercial ELISA kit and the electrochemical assay. In conclusion, with the electrochemical immunoassay, it was possible to distinguish among the serum samples from ill or healthy subjects.

4. Conclusions

An electrochemical assay based on antibody-coated magnetic beads and disposable electrochemical sensors was developed and applied to the detection of neopterin in serum samples. The device was simple and cost-effective, since it involved low amounts of reagents and low-cost mass-produced sensors.

The developed assay was based on a competitive scheme in which monoclonal and polyclonal antibodies were employed as capture elements. The performance of the assay in terms of sensitivity, reproducibility and selectivity was studied in buffer and in serum. The calibration curve covered an appropriate area to distinguish between ill and healthy subjects. The LOD values with mAb and pAb were 0.5 and 0.008 ng/mL, respectively and the average coefficient of variation (ACV) resulted 10%. The LOD found was even better with respect to that reported by classical ELISA performed with the same antibodies and it was much lower than the clinically useful cut-off value (3.04 ng/mL).

Finally, the proposed method was applied to the analysis of some clinical samples and it resulted as a promising tool to predict the risk of a possible disease related with neopterin concentrations.

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