Analytical Biochemistry 380 (2008) 262–267

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Microfluidic immunosensor design for the quantification of interleukin-6 in human serum samples

Germán A. Messina, Nancy V. Panini, Noelia A. Martinez, Julio Raba *

INQUISAL, Department of Chemistry, National University of San Luis, CONICET, Chacabuco y Pedernera, D5700BWS, San Luis, Argentina

ARTICLE INFO

Article history:
Received 1 April 2008
Available online 10 June 2008

Keywords:
Enzyme immunoassays
Interleukin-6
Amperometric immunosensor
Alkaline phosphatase
Microfluidic
Flow injection analysis

ABSTRACT

Interleukin-6 (IL-6), an inflammatory cytokine, is one of the most important mediators of fever, the acute phase response, and inflammatory conditions. Described here is an integrated microfluidic immunosensor capable of detecting the concentration of IL-6 in human serum samples by use of an electrochemical method in a microfluidic biochip format. The detection of IL-6 was carried out using a sandwich immunoassay method based on the use of anti-IL-6 monoclonal antibodies, immobilized on a 3-aminopropyl-modified controlled-pore glass (APCPG) packet in a central channel (CC) of the microfluidic system. The IL-6 in the serum sample is allowed to react immunologically with the immobilized anti-IL-6 and biotin-labeled second antibodies specific to IL-6. After washing, the streptavidin–alkaline phosphatase conjugate is added. *p*-Aminophenyl phosphate is converted to *p*-aminophenol by alkaline phosphatase, and the electroactive product is quantified on a gold electrode at 0.10 V. For electrochemical detection and enzyme immunoassay, the LOD was 0.41 and 1.56 pg mL^{-1}, respectively. Reproducibility assays employed repetitive standards of IL-6, and the intra- and inter-assay coefficients of variation were below 6.5%. Compared with the traditional IL-6 sensing method, the integrated microfluidic immunosensor required smaller amounts of sample to perform faster detection.

© 2008 Elsevier Inc. All rights reserved.

Interleukin-6 (IL-6)[1], a pleiotropic cytokine that has a critical role in the inflammatory response, has been implicated in the pathogenesis of a number of inflammatory conditions, such as psoriasis, rheumatoid arthritis, cardiovascular disease, and inflammatory bowel disease [1–5]. In addition, much evidence indicates a key role for IL-6 in lymphoproliferative conditions. In particular, IL-6 has been shown to be a growth factor for multiple myeloma cells [6,7]. B-cell lymphomas also produce high levels of IL-6, which in fact represents an important growth factor in at least some forms of this pathology. In addition, the presence of blasts in patients with B-cell lymphoma has been shown to correlate with IL-6 production [8].

IL-6 elicits B cells to undergo proliferation and differentiation into antibody-forming cells and assists in IL-4-dependent IgE synthesis and T-cell activation, growth, and differentiation. IL-6 also acts in conjunction with IL-3 to induce the proliferation of pluripotent hematopoietic progenitors [9]. As an important member of the cytokine network, IL-6 mediates the acute phase response in the liver and stimulates the production of C-reactive protein (CRP) and fibrinogen. A variety of infectious diseases can cause an increase in serum IL-6 level, and increased IL-6 is, in turn, closely associated with mortality [10,11].

A great deal of evidence indicates that this cytokine is also a growth factor for B lymphocytes immortalized with the Epstein–Barr virus [12], and its production has been demonstrated, especially in elderly people [13]. B-cell lymphoproliferative diseases associated with Epstein–Barr virus infection may result from rare but serious complications after organ or bone marrow transplantation [14]. Normal serum IL-6 levels are usually less than 4 pg/mL [15,16].

Thus, the determination of IL-6 levels is very useful to clinical diagnosis. Various commonly available methods have been developed for determination of IL-6, such as radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), immunoaffinity column assay, and fluorometric, electrochemical, and capacitive determinations [17–22]. These laboratory techniques, unfortunately, require highly qualified personnel, tedious assay time, or sophisticated instrumentation. Therefore, development of a new method with high sensitivity and specificity for direct detection of IL-6 is highly desirable.

One possible solution involves the use of microbiochips that employ microfluidics. These kinds of devices that use microelectromechanical systems (MEMS) technology have been developed in

* Corresponding author. Fax: +54 2652 43 0224.
E-mail address: jraba@unsl.edu.ar (J. Raba).

[1] *Abbreviations used:* IL-6, interleukin-6; ELISA, enzyme-linked immunosorbent assay; FI, flow injection; APCPG, 3-aminopropyl-modified controlled-pore glass; pAPP, *p*-aminophenyl phosphate; pAP, *p*-aminophenol; CC, central channel; pNPP, 4-nitrophenyl phosphate disodium salt hexahydrate; PBS, phosphate-buffered saline; QI, *p*-benzoquinoneimine; DEA buffer, 100 mM diethanolamine/50 mM KCl/1 mM $MgCl_2$, pH 9.6.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.05.055

the last decade, and include lab-on-a-chip [23], biosensors [24,25], and a cell handling system [26]. Recently, these microfluidic systems have been integrated with biosensing devices to perform ELISA [27], electrochemical sensing [28], DNA detection [29,30], cell detection [31], and many procedures.

Based on the results of the studies conducted to date, microfluidic biosensors are likely to improve analytical efficiency by reducing the sample volume required and the time required for analysis while increasing sensitivity and enabling processing of multiple samples via automation [32,33].

Heterogeneous enzyme immunoassays, coupled with a flow injection (FI) system and electrochemical detection, represent a powerful analytical tool for the determination of low levels of many analytes such as antibodies, hormones, drugs, tumor markers, and viruses [34].

Recently, electrochemical immunosensors are attracting increasing attention because they combine the high specificity of traditional immunoassay methods with the low detection limits and low expense of electrochemical measurement systems [35]. The methods that employ immunosensors are very rapid and have both high specificity and sensitivity [36]. In addition, they have the advantage of requiring small sample volumes, affording an increase in the number of samples analyzed and, thus, reducing costs when compared with the conventional analytical methods.

In the work described in this article, we coupled a microfluidic immunosensor to a gold electrode for rapid and sensitive quantification of IL-6 in human serum samples. Detection of IL-6 was carried out using a sandwich immunoassay method based on the use of anti-IL-6 monoclonal antibodies immobilized on 3-aminopropyl-modified controlled-pore glass (APCPG). The IL-6 in the serum sample is allowed to react immunologically with the immobilized anti-IL-6 and biotin-labeled second antibodies specific to IL-6. After washing, enzyme (streptavidin–alkaline phosphatase conjugate) is added. *p*-Aminophenyl phosphate (pAPP) is converted to *p*-aminophenol (pAP) by alkaline phosphatase and the electroactive product is quantified on a gold electrode at 0.10 V. The current resulting from oxidation of the product of the enzymatic reaction is proportional to the activity of the enzyme and, consequently, to the amount of IL-6 bound to the surface of the immunosensor.

This method allows for rapid determination of IL-6, minimizes waste of expensive antibodies and other reagents, and does not require highly skilled technicians or expensive and dedicated equipment.

Materials and methods

Reagents and solutions

All reagents used were of analytical reagent grade. Mouse monoclonal IL-6 antibody (ab9324) and goat polyclonal biotin-conjugated IL-6 antibody (ab17529) were supplied by Abcam Inc., Cambridge, MA, USA. Streptavidin–alkaline phosphatase conjugate was purchased from Sigma Chemical Company, St. Louis, MO, USA. Glutaraldehyde (25% aqueous solution) was purchased from Merck, Darmstadt, Germany. The serum samples were obtained in clinical laboratories of San Luis City, Argentina. APCPG (1400 Å in mean pore diameter and 24 $m^2\ mg^{-1}$ in surface area) was from Electro Nucleonics (Fairfield, NJ, USA) and contained 48.2 $\mu mol\ g^{-1}$ amino groups. 4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP) was purchased from Fluka Chemie (Steinheim, Switzerland). All other reagents employed were of analytical grade and used without further purifications. Aqueous solutions were prepared using purified water from a Milli-Q system. The ChemiKine Human Interleukin-6 (IL-6) Sandwich ELISA Kit for the quantitative determination of IL-6 was purchased from Chemicon International, Inc. USA & Canada and was used in accordance with the manufacturer's instructions [37].

Flow-through reactor/detector unit

The main body of the sensor was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the microfluidic immunosensor and the detector system. The gold electrode is at the end of the central channel (CC). Typically, the CC carried 0.3 mg of controlled-pore glass, and the end of the CC was blocked with glass fibers. The diameter of the CC was 150 μm and the diameter of the accessory channels was 100 μm. All solutions and reagent temperatures were conditioned before the experiment using a Vicking Masson II laboratory water bath (Vicking SRL, Buenos Aires, Argentina).

Amperometric detection was performed using the BAS LC-4 C (Bioanalytical Systems, West Lafayette, IN, USA). The BAS 100B electrochemical analyzer Bioanalytical Systems) was used for cyclic voltammetric analysis. The potential applied to the gold electrode was 0.10 V versus the Ag/AgCl wire pseudo-reference electrode and a Pt wire was the counterelectrode . At this potential, a catalytic current was well established. Pumps (Baby Bee Syringe Pump, Bioanalytical Systems) were used for pumping, sample introduction, and stopping flow.

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.). Absorbance was determined with a Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 General UV/VIS spectrophotometer.

ELISA for determination of IL-6

An IL-6 standard was supplied with the ChemiKine Human IL-6 Sandwich ELISA Kit. A standard curve for the spectrophotometric procedure was produced by following the manufacturer's protocol with a range of detection of 0 to 500 pg mL^{-1}. Concentrations of IL-6 were detected spectrophotometrically by measuring absorbance changes at 490 nm [37].

Synthesis of pAPP

Synthesis of pAPP by catalytic hydrogenation of pNPP was performed using the procedure described in Ref. [38] with the following modifications. In a 100-mL glass hydrogenation vessel, 2.00 g of pNPP was dissolved in 30 ml of 50% ethanol containing 0.11 g of 10% palladium on charcoal catalyst. The hydrogenation reaction

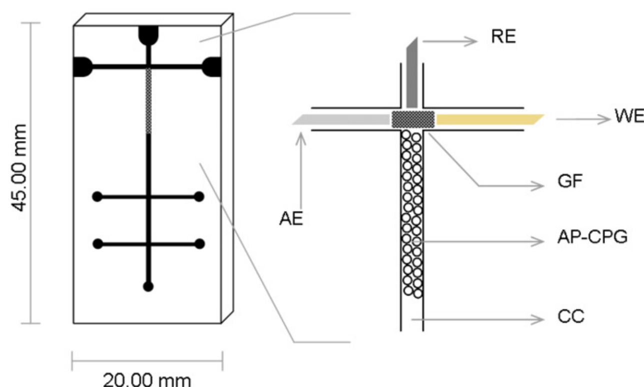

Fig. 1. Schematic representation of microfluidic immunosensor. RE, reference electrode; AE, gold electrode; WE, wire electrode; RD, rotating disk; GF, glass fiber; APCPG, 3-aminopropyl-modified controlled-pore glass; CC, central channel. All measurements are given in millimeters.

was conducted overnight at room temperature at an initial pressure of 1.3 atm. The resultant mixture was filtered on a Buchner funnel to remove the catalyst, and the volume of solvent was reduced to 10 mL using a rotary evaporator. The oily residue was diluted to 20 mL with distilled. deionized water and clarified by filtration. Cold ethanol (20 mL, 4 °C) was added to the filtrate, and the precipitated product was recovered by filtration, dried under vacuum, and stored at −10 °C. The pAPP product was greater than 98% pure as determined by NMR and electrochemical methods.

Immunosensor preparation

Mouse monoclonal IL-6 antibody was immobilizing on APCPG in a Eppendorf tube. The APCPG was allowed to react with an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After washes with purified water and 0.10 M phosphate buffer, pH 7.00, 25 μL of antibody preparation (10 μg mL^{-1} 0.01 M PBS, pH 7.2) was coupled to the residual aldehyde groups overnight at 5 °C. The immobilized antibodies preparation was finally washed with PBS (pH 7.00) and stored in the same buffer at 5 °C. The immunsensor was prepared by packing 0.3 mg of modified APCPG in the CC. Immobilized antibody preparations were perfectly stable for at least 1 month.

Amperometric analysis of IL-6 in human serum samples

This method was applied to determine IL-6 in 24 human serum samples. The carrier buffer was 0.01 M PBS, pH 7.2, with 0.5% skim milk to block the unspecific binding.

The serum samples were diluted fourfold with a solution of the biotinylated goat anti-IL-6 polyclonal antibody at a concentration of 0.15 μg/ml (0.01 M PBS, pH 7.2) and then injected into the PBS carrier stream at a flow rate of 5 μL min^{-1} for 5 min at 25 °C. The immunosensor was washed with 0.01 M PBS, pH 7.2, at a flow rate of 5 μL min^{-1}. After washing, the streptavidin–alkaline phosphatase conjugate (2.23 U/mL) was injected at a flow rate of 5 μL min^{-1} for 5 min. The immunosensor was then washed free of any traces of unbound enzyme conjugate with 0.01 M PBS, pH 7.2. DEA buffer (100 mM diethanolamine, 50 mM KCl, 1 mM $MgCl_2$, pH 9.6) was used to prepare the pAPP solution. Five microliters of substrate solution (2.7 mM pAPP in DEA buffer, pH 9.6) was injected into the carrier stream at a flow rate of 5 μL min^{-1}, and the enzymatic product (pAP) was measured on the surface of a gold electrode at the end of the CC. For the next analysis, the immunosensor was conditioned with a flow of 50 μL of desorption buffer (0.1 M glycine–HCl, pH 2) and then washed with 50 μL of 0.01 M PBS, pH 7.2, at a flow rate of 5 μL min^{-1}.

A standard curve for the amperometric procedure was produced by following our protocol with a sequence of standard dilutions that covered the clinical range 0–400 pg mL^{-1} IL-6, supplied with the ChemiKine Human IL-6 Sandwich ELISA Kit. Amperometric measurements were performed at 0.10 V at room temperature in DEA buffer, pH 9.6, and the resulting anodic current was displayed on the *x*–*y* recorder. When not in use, the immunosensor was stored in 0.01 M PBS, pH 7.2, containing sodium azide (0.01%) at 4 °C. The stock solution of pAPP was prepared freshly before the experiment and stored in the dark for the duration of the experiment.

Results and discussion

Electrochemical study of pAP with the gold electrode

The electrochemical behavior of the hydrolysis product (pAP) of the enzyme substrate pAPP was examined by cyclic voltammetry at the gold electrode. A cyclic voltammetric study of 1.0×10^{-3} mol L^{-1} of pAP in DEA buffer, pH 9.6, was performed by scanning the potential from –300 to 500 mV versus Ag/AgCl. The cyclic voltammogram showed a well-defined anodic peak and a corresponding cathodic peak, which corresponds to the transformation of pAP to *p*-benzoquinoneimine (QI) and vice versa in a quasi-reversible two-electron process (Fig. 2). A peak current ratio (I_{C1}/I_{A1}) of nearly unity, particularly during the recycling of potential, can be considered a criterion for the stability of QI produced at the surface of electrode under experimental conditions.

Optimum conditions for the immune reactions and the determination of enzymatic products

To optimize the proposed method, an understanding of the effects of the parameters governing the system is necessary. It has been shown that the theoretical framework developed for a static ELISA system cannot be used to describe the kinetics of antibody–antigen interactions occurring in a continuous-flow immunoassay [39]. Several parameters differ significantly. Buffer flow reduces the limitations of diffusion as observed in static ELISA systems [40]. Furthermore, the surface density of immobilized antigens in the flow immunoassay is at least three orders of magnitude higher than that in static ELISA systems, because the controlled-pore grass increases the area for immobilization about three orders of magnitude [41].

The proposed method manifolds follow enzyme immunoassay principles, but instead of using a microtiter plate, the reagents and washing buffers were pumped consecutively through the microfluidic immunosensor containing immobilized antibodies, coupled to electrochemically detection for the determination of IL-6 in human serum samples.

Microfluidic control systems are essential for the control of a minute volume of fluid because of their rapid and precise control features; therefore, in our microbiosensor, all reactions and washing procedures were performed using a syringe pump. The flow rates of the sample and reagent have an effect on the reaction efficiency of the antigen–antibody interactions, and unlike in conventional immunoassays, samples and reagents in our system are continuously flowing through the microbiochips. The optimal flow rate was determined by analyzing 100 μL of an IL-6 standard at different flow rates and evaluating the current generated during the immune reaction. As shown in Fig. 3, flow rates from 1 to 5 μL min^{-1} had little effect on antigen–antibody reaction. Con-

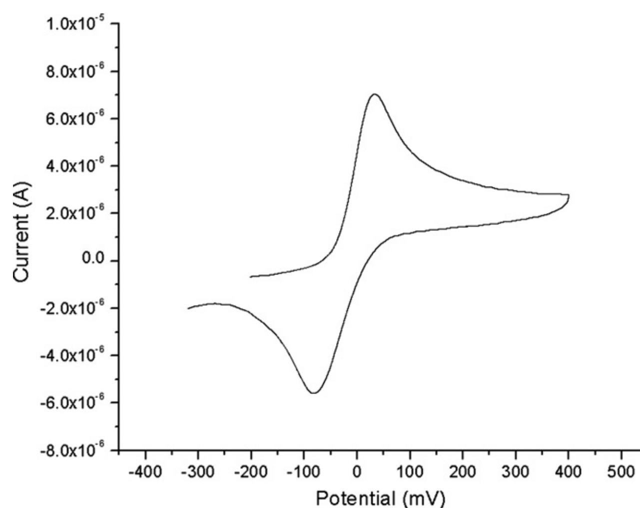

Fig. 2. Cyclic voltammograms of pAP in aqueous solution containing 1.0×10^{-3} mol L^{-1} pAP in DEA buffer, pH 9.6, with a gold electrode. Scan rate: 100 mV s^{-1}.

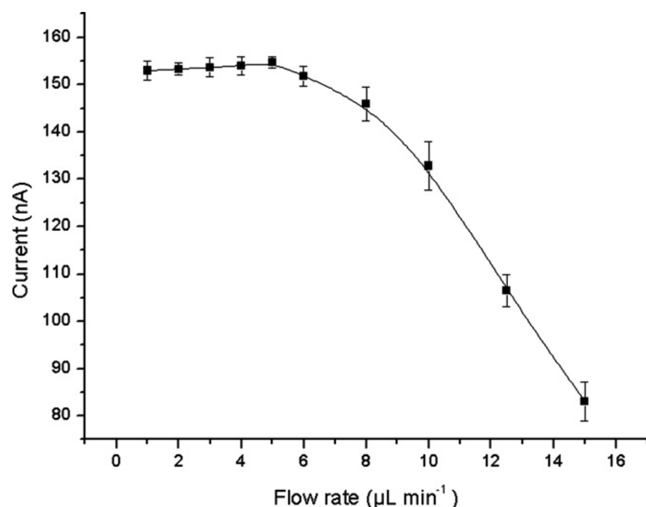

Fig. 3. Effect of flow rates from 1 to 15 μL min^{-1} on analysis of 100 μL of IL-6 standard.

versely, when the flow rate exceeded 8 μL min^{-1}, the signal was dramatically reduced. Therefore, a flow rate of 5 μL min^{-1} was used for injections of reagents and washing buffer.

The response current obtained from the product of enzymatic reaction is proportional to the activity of the enzyme conjugated and, consequently, to the amount of IL-6 in serum samples bound to APCPG modified with IL-6-specific antibodies.

Sample volume was in the range 10 to 40 μL. Sensitivity almost tripled in the range 10 to 25 μL. Insignificant differences were observed with larger sample volumes. A sample volume of 25 μl was used to evaluate other parameters.

The rate of enzymatic response under flow conditions was studied in the pH range 8–10 and reached a maximum at pH 9.6. The pH value used was 9.6 in DEA buffer. The effect of varying pAPP concentration from 0.1 to 5 mM on the enzymatic response was evaluated. The optimum pAPP concentration determined, 2.7 mM, was then used.

Quantitative test for the detection of IL-6 in the microfluidic immunosensor

Under the selected conditions described above, the electrochemical response of the enzymatic product is proportional to the concentration of IL-6 in the serum. Table 1 summarizes the complete analytical procedure required for the IL-6 immunoassay using our system; the total time required for the immunoassay was found to be approximately 25 min.

A linear calibration curve to predict the concentration IL-6 in serum was produced within the range 0–400 pg mL^{-1} using an IL-6 standard supplied with the IL-6 Sandwich ELISA Test kit. The linear regression equation was $i = 7.71584 + 1.54* C_{IL\text{-}6}$, with the linear regression coefficient $r = 0.998$. The coefficient of variation (CV) for the determination of 100 pg mL^{-1} IL-6 was below 4.3% (six replicates). These values demonstrate that our microfluidic immunosensor can be used to quantify the amount of IL-6 in unknown samples.

An EIA was also carried out as described, absorbance changes were plotted against the corresponding IL-6 concentration, and a calibration curve was constructed. The linear regression equation was $A = -0.01423 + 0.0052* C_{IL\text{-}6}$, with the linear regression coefficient $r = 0.996$, and the CV for the determination of 100 pg mL^{-1} IL-6 was 8.3% (six replicates).

The detection limit (LOD) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For electrochemical detection and EIA, the LODs were 0.41 and 1.56 pg mL^{-1}, respectively. This result shows that electrochemical detection was more sensitive than the spectrophotometric method.

Sensitivity (S) is defined as the slope of the regression line of the signal-versus-concentration plot. For electrochemical detection and EIA, S values were 1.54 nA/pg mL^{-1} and 0.0052 Abs/pg mL^{-1}, respectively.

The precision of the electrochemical assay configured CV was checked with control serum at IL-6 concentrations of 20, 200, and 400 pg mL^{-1}. The within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for 3 consecutive days to estimate between-assay precision. The results obtained are summarized in Table 2. The IL-6 assay showed good precision; the CV within-assay values were below 4.3% and the between-assay values below 6.5%. Reproducibility assays were done using a repetitive standard ($n = 6$) of 100 pg mL^{-1} IL-6 (Table 3).

Total assay time, including injection and detection steps, for the IL-6 measurements was less than 25 min. For the next analysis, the immunosensor was regenerated by injection of desorption buffer (0.1 M glycine–HCl, pH 2) and then washed with 0.01 M PBS (pH 7.2); in this manner, our system could be used more than 100 determinations.

The electrochemical system was compared with a commercial spectrophotometric system for the quantification of IL-6 in serum samples. The slopes obtained were reasonably close to 1, indicating

Table 1
Sequences required for the IL-6 immunoassay

Sequence	Condition	Time (min)
Antigen	IL-6 diluted 4-fold with a polyclonal biotin- conjugated IL-6 antibody, 5 μL min^{-1}	5
Washing buffer	Flow rate: 5 μL min^{-1} (PBS, pH 7.2)	3
Conjugated enzyme	Streptavidin–alkaline phosphatase conjugated (2.23 U/mL), 5 μL min^{-1}	5
Washing buffer	Flow rate: 5 μL min^{-1} (PBS, pH 7.2)	3
Substrate	2.7 mM p-APP in a DEA buffer, pH 9.6, 5 μL min^{-1}	5
Signal analysis	LC-4C amperometric detector, 0.10 V	2

Table 2
Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control serum, repeated 3 consecutive days)

Control serum[a] (pg mL^{-1} IL-6)	Within-assay precision		Between-assay precision	
	Mean	CV (%)	Mean	CV (%)
20	20.93	3.61	21.61	4.91
200	204.47	2.74	211.04	5.62
400	393.75	4,26	387.13	6.41

[a] Dilutions of the IL-6 standard.

Table 3
Reproducibility assays using repetitive standards ($n = 6$) of 100 pg mL^{-1} IL-6

100 pg mL^{-1} IL-6 standard	Proposed method (pg mL^{-1})	ELISA (pg mL^{-1})
1	103.23	98.33
2	99.15	102.73
3	98.23	97.45
4	102.93	96.26
5	100.87	107.96
6	98.68	104.63
Mean ± SD	100.51 ± 2.18	101.22 ± 4.62

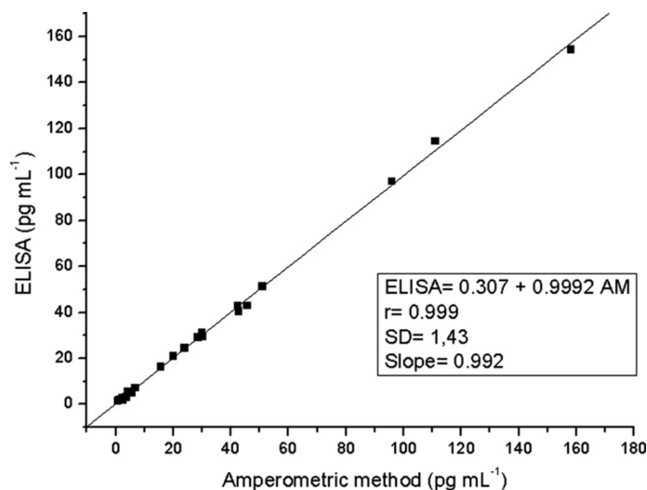

Fig. 4. Correlation between proposed method and commercial photometric assays.

good correspondence between the two methods (Fig. 4). Compared with ELISA, our method shows large enhancement in sensitivity. These results suggest that the concentration of IL-6 detectable with this system is at the levels of clinical analysis, and its sensitivity is high enough to determine IL-6 in serum of patients with very low levels.

Conclusions

A microfluidic immunosensor coupled with a flow injection system for rapid, sensitive and selective quantification of IL-6 in serum, even in patients with very low levels, was developed using electrochemical detection.

The overall assay time (25 min) was much less than the 5 hours normally used with conventional batch well ELISA, which is more than 12 times faster than the plate method, with no reduction in selectivity. Our method also minimizes the waste of expensive reagents, is physically and chemically stable, has a low background current and wide working potential range, and is accurate.

We took advantage of the simplicity of ELISA to construct an immunosensor that was capable of measuring the same levels of IL-6 in human serum sample as detected with conventional methods while having the advantages of low detection limit, speed, and simplicity. Owing to the wider applications in many fields, miniaturized immunosensors will make a significant contribution to faster, direct, and secure analysis of chemicals, pathogens, and biological molecules.

Acknowledgments

The authors thank the Universidad Nacional de San Luis and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for financial support.

References

[1] J. Van Snick, Interleukin-6: an overview, Annu. Rev. Immunol. 8 (1990) 253–278.
[2] K. Ishihara, T. Hirano, IL-6 in autoimmune disease and chronic inflammatory proliferative disease, Cyto. Growth. Factor Rev. 13 (2002) 357–368.
[3] T. Hosokawa, K. Kusugami, K. Ina, T. Ando, M. Shinoda, A. Imada, M. Ohsuga, T. Sakai, T. Matsuura, K. Ito, K. Kaneshiro, Interleukin-6 and soluble interleukin-6 receptor in the colonic mucosa of inflammatory bowel disease, J. Gastroenterol. Hepatol. 14 (1999) 987–996.
[4] R. Glaser, J.K. Kiecolt-Glaser, R.H. Bonneau, W. Malarkery, S. Kennedy, J. Hughes, Stress-induce modulation of the immune response to recombinant hepatitis B vaccine, Psychosom. Med. 109 (1992) 540–542.
[5] G.A. Roselle, C.L. Mendenhall, Alteration of in vitro human lymphocyte function by ethanol, acetaldehyde and acetate, J. Clin. Lab. Immunol. 9 (1982) 33–37.
[6] V.M. Lauta, Interleukin-6 and the network of several cytokines in multiple myeloma: an overview of clinical and experimental data, Cytokine 16 (2001) 79–86.
[7] M.A. Frassanito, A. Cusmai, G. Iodice, F. Dammacco, Autocrine interleukin-6 production and highly malignant multiple myeloma: relation with resistance to drug-induced apoptosis, Blood 97 (2001) 483–489.
[8] D. Emilie, J. Coumbaras, M. Raphael, O. Devergne, H.J. Delecluse, C. Gisselbrecht, Interleukin-6 production in high-grade B lymphomas: correlation with the presence of malignant immunoblasts in acquired immunodeficiency syndrome and in human immunodeficiency virus-seronegative patients, Blood 80 (1992) 498–504.
[9] A.H. Swiergiel, A.J. Dunn, Feeding, exploratory, anxiety- and depression-related behaviors are not altered in interleukin-6-deficient male mice, Behav. Brain Res. 171 (2006) 94–108.
[10] L.C. Casey, R.A. Balk, R.C. Bone, Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome, Ann. Intern. Med. 119 (1993) 771–778.
[11] H. Shin, Y. Lee, Y. Chang, J. Lee, B. Kim, K. Min, Y. Kim, Suppression of interleukin-6 production in macrophages by furonaphthoquinone NFD-37, Int. Immunopharmacol. 6 (2006) 916–923.
[12] A. Durandy, D. Emilie, M. Peuchmaur, M. Forveille, C. Clement, J. Wijdenes, Role of IL-6 in promoting growth of human EBV-induced B-cell tumors in severe combined immunodeficient mice, J Immunol. 152 (1994) 5361–5367.
[13] F. Olivieri, M. Bonafe, S. Giovagnetti, R. Stecconi, M. Cardelli, L. Cavallone, In vitro IL-6 production by EBV-immortalized B lymphocytes from young and elderly people genotyped for 174 C/G polymorphism in IL-6 gene: a model to study the genetic basis of inflammation, Mech. Ageing Dev. 124 (2003) 549–553.
[14] R.E. Curtis, L.B. Travis, P.A. Rowlings, G. Socie, D.W. Kingma, P.M. Banks, Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood 94 (1999) 2208–2216.
[15] H.C. Nousari, A. Kimyai-Asadi, G.J. Anhalt, Elevated serum levels of interleukin-6 in paraneoplastic pemphigus, J. Invest. Dermatol. 112 (1999) 396–397.
[16] J.M. Marugán Miguelsanz1, M.A. Suárez Rodríguez, L.M. Rodríguez Fernández1, J.M. García Ruiz de Morales, Niveles normales de interleukinas 6 y 8 en suero y orina de niños sanos asintomáticos, Bol. Soc. Can. Ped. 30 (2006) 47–55.
[17] H. Brailly, F.A. Montero-Julian, C.E. Zuber, S. Flavetta, J. Grassi, F. Houssiau, J. VanSnick, Total interleukin-6 in plasma measured by immunoassay, Clin. Chem. 40 (1994) 116–123.
[18] L.M. Lambros, E. Bathrellos, S. Evriklia, C. Penelope, A. Ioannou, A highly sensitive enzyme-amplified lanthanide luminescence immunoassay for interleukin-6, Clin. Chem. 44 (1998) 1351–1353.
[19] T. Krakauer, A sensitive,specific immunobioassay for quantitation of human interleukin 6, J. Immunoassay 14 (1993) 267–277.
[20] K.Z. Liang, W.J. Mub, Flow-injection immuno-bioassay for interleukin-6 in humans based on gold nanoparticles modified screen-printed graphite electrodes, Anal. Chim. Acta 580 (2006) 128–135.
[21] C. Berggren, B. Bjarnason, G. Johansson, An immunological interleukine-6 capacitive biosensor using perturbation with a potentiostatic step, Biosens. Bioelectron. 13 (1998) 1061–1068.
[22] F. Deckert, F. Legay, Development and validation of an IL-6 immuno-receptor assay based on surface plasmon resonance, J. Pharm. Biomed. Anal. 23 (2000) 403–412.
[23] J.H. Cho, S.M. Han, E.H. Paek, I.H. Cho, S.H. Paek, Plastic ELISA-on-a-chip based on sequential cross-flow chromatography, Anal. Chem. 78 (2006) 793–800.
[24] K. Lange, G. Blaess, A. Voigt, R. Gotzen, M. Rapp, Integration of a surface acoustic wave biosensor in a microfluidic polymer chip, Biosens. Bioelectron. 22 (2006) 227–232.
[25] N.V. Zaytseva, V.N. Goral, R.A. Montagna, A.J. Baeumner, Development of a microfluidic biosensor module for pathogen detection, Lab on a Chip 5 (2005) 805–811.
[26] K.R. King, S. Wang, D. Irimia, A. Jayaraman, M. Toner, M.L. Yarmush, A high-throughput microfluidic real-time gene expression living cell array, Lab on a Chip 7 (2007) 77–85.
[27] C.H. Wang, G.B. Lee, Automatic bio-sampling chips integrated with micro-pumps and micro-valves for multiple disease detection, Biosens. Bioelectron. 21 (2005) 419–425.
[28] C.F. Lin, G.B. Lee, C.H. Wang, H.H. Lee, W.Y. Liao, T.C. Chou, Microfluidic pH-sensing chips integrated with pneumatic fluid-control devices, Biosens. Bioelectron. 21 (2006) 1468–1475.
[29] C.S. Liao, G.B. Lee, H.S. Liu, T.M. Hsieh, C.H. Luo, Miniature RT-PCR system for diagnosis of RNA-based viruses, Nucleic Acids Res. 33 (2005) 156–162.
[30] K.Z. Liang, W.J. Mu, Flow-injection immuno-bioassay for interleukin-6 in humans based on gold nanoparticles modified screen-printed graphite electrodes, Anal. Chim. Acta 580 (2006) 128–135.
[31] C.H. Lin, G.B. Lee, Micromachined flow cytometers with embedded etched optic fibers for optical detection, J. Micromech. Microeng. 13 (2003) 447–453.
[32] K.S. Kim, J.K. Park, Magnetic force-based multiplexed immunoassay using superparamagnetic nanoparticles in microfluidic channel, Lab on a Chip 5 (2005) 657–664.

[33] S.C. Tadic, G. Dernick, D. Juncker, G. Buurman, H. Kropshofer, B. Michel, C. Fattinger, E. Delamarche, High-sensitivity miniaturized immunoassays for tumor necrosis factor α using microfluidic systems, Lab on a Chip 4 (2004) 563–569.

[34] G. Gübitz, C. Shellum, Flow injection immunoassays, Anal. Chim. Acta 283 (1993) 421–428.

[35] L. Shen, P. He, An electrochemical immunosensor based on agarose hydrogel films for rapid determination of ractopamine, Electrochem. Commun. 9 (2007) 657–662.

[36] C.S. Riccardi, P.I. Costa, H. Yamanaka, Imunossensor amperometrico, Quim. Nova 25 (2002) 316–320.

[37] Instruction Manual for ChemiKine™ Human Interleukin-6 (IL-6) Sandwich ELISA Kit for the Quantitative Determination of IL-6, CHEMICON International, Inc. USA & Canada, 2004.

[38] A.G. Gehring, C.G. Crawford, R.S. Mazenko, L.J. Van Houten, J.D. Brewster, Enzyme-linked immunomagnetic electrochemical detection of *Salmonella typhimurium*, J. Immunol. Methods 195 (1996) 15–25.

[39] G.A. Wemhoff, S.Y. Rabbany, A.W. Kusterbeck, R.A. Ogert, R. Bredehorst, F.S. Ligler, Kinetics of antibody binding at solid-liquid interfaces in flow, J. Immunol. Methods 156 (1992) 223–230.

[40] N.V. Panini, G.A. Messina, E. Salinas, H. Fernández, J. Raba, Integrated microfluidic systems with an immunosensor modified with carbon nanotubes for detection of prostate specific antigen (PSA) in human serum samples, Biosens. Bioelectron. 23 (2008) 1145–1151.

[41] M.C. Gosnell, R.E. Snelling, H.A. Mottola, Construction and performance of plastic-embedded controlled-pore glass open tubular reactors for use in continuous-flow systems, Anal. Chem. 58 (1986) 1585–1587.