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

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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.

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

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[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.

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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

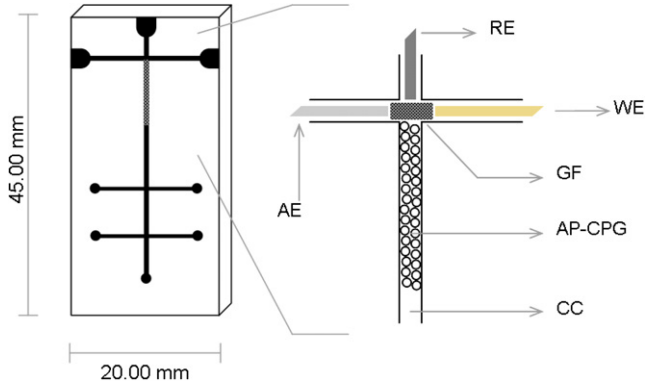

Fig. 1. Schematic representation of microfluidic immunosensor. RE, reference electrode; AE, gold electrode; WE, wire electrode; RD, rotating disk; GF, glass fiber; APCPG, 3-aminopropyl-modified controlled-pore glass; CC, central channel. All measurements are given in millimeters.