Electrochemical impedance spectroscopy: An effective tool for a fast microbiological diagnosis

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ABSTRACT

Dielectric spectroscopy, also called electrochemical impedance spectroscopy, is traditionally used in monitoring corrosion and electro-deposition processes in the coating and characterization assessment of many kinds of sensors and semi-conductors. Its application in biotechnology for the characterization of cell cultures has, however, been notably expanded in the last decade. As a transductional principle, impedance has been applied in the field of microbiology as a means of detecting and quantifying pathogenic bacteria. This paper reviews the state-of-the-art of Impedance Microbiology, its progress and its applications for the detection of foodborne pathogenic bacteria, including the use of interdigitated microelectrodes, the development of chip-based impedance microbiology and the integration of impedance biosensors along with other techniques such as dielectrophoresis and electropermeabilization. Reference is made to basic components, definitions and principles of this technique, as well as to the explanation of the components and principles for cell culture design and the use of equivalent circuits for the analysis of the systems based on this alternative.

Keywords: bacteria detection, diagnosis, impedance, microbiology

RESUMEN

Espectroscopía de impedancia electroquímica, herramienta eficaz para el diagnóstico rápido microbiológico. La espectroscopia dieléctrica o espectroscopia de impedancia electroquímica es empleada tradicionalmente en el registro y estudio de los procesos de corrosión y electrodeposición, en la evaluación de recubrimientos y en la caracterización de muchos tipos de sensores y semiconductores. En la última década se han ampliado notablemente sus aplicaciones en la Biotecnología para la caracterización de células biológicas, el diagnóstico de enfermedades y la caracterización del cultivo de células. Como principio de transducción, la técnica de impedancia ha sido aplicada en el campo de la microbiología como un medio para detectar y cuantificar microorganismos patógenos. El presente trabajo revisa el estado del arte de la microbiología de impedancia, el progreso y las aplicaciones en la detección de microorganismos patógenos incluido el uso de los microelectrodos interdigitados, el desarrollo de la miniaturización de la microbiología de impedancia y la integración de los biosensores de impedancia con otras técnicas como la dielectroforesis y la electropermeabilización. Se hace referencia a conceptos básicos, definiciones y fundamentos de esta técnica, así como se abordan los componentes, principios para el diseño del medio de cultivo y uso de circuitos equivalentes para el análisis de los sistemas basados en esta alternativa.

Palabras clave: detección de bacterias, diagnóstico, impedancia, microbiología

Introduction

The growing use of electrochemical sensors in environmental applications, the industry and in the medical field has brought about the urging need to understand surface properties and the important analytical systems as a whole. This seems to indicate that the scientific community has chosen impedance spectroscopy to characterize a large number of electrochemical systems. The information obtained from this technique has been used to make electrochemical sensors having excellent properties (linearity, thermal and temporal stability, etc).

Impedance spectroscopy is a powerful tool for a fast biomolecule diagnosis and for analyses in cell cultures. Its superiority over other laboratory techniques lies in that it uses a small signal, generally in the tension mode, thus minimizing the alterations of the properties of the medium, in other words, applied stimulation does not alter the equilibrium conditions of the system.

The signal applied to the samples makes it possible to link the properties of the liquid or solid being studied with the variations or changes obtained in its characteristic impedance. This is due to the physical structure of the material, to the chemical processes occurring in it, or to a combination of both. Consequently, electrochemical impedance spectroscopy is a non-destructive technique providing robust measurements.

Classical impedance microbiology: Definition and basic concepts

Electric impedance as a transduction principle has been applied to a great variety of biological, physiological and medical problems [1, 2].

Before going into the topic, it is important to analyze some basic concepts. It is widely known that electric resistance R is the ability of an element from the circuit to resist the flow of electric current. Ohm's Law defines

1. Geddes LA, Baker LE. Principles of applied biomedical instrumentation. New York: John Wiley & Sons. 1989.

2. Eden G, Eden R. Enumeration of microorganisms by their dynamic AC conductance patterns. IEEE Trans Biomed Eng. 1984;31(2):93-8.

the resistance as the relation between the tension and the electric current.

$$R = \frac{V}{I} \qquad (1)$$

Where:

V corresponds to the value of tension, and *I* to that of the electric current.

The use of equation 1 is limited only to an element of the circuit: the ideal resistor, which has several determining factors: 1) it fulfills Ohm's Law in all levels of tension and current, 2) its resistance value is not related to frequency, and 3) signals of tension and current through a resistor are in the same phase.

The real world has elements with a much more complex behavior. These elements make us reject the simple concept of resistance. As a result, impedance is used instead, which is a parameter of a much more general circuit. As with resistance, impedance is a measurement of the ability of a circuit to resist the flow of the electric current. Unlike resistance, impedance is not limited by any of the above mentioned determining factors.

Electrochemical impedance is usually obtained by applying a potential of alternating current to an electrochemical cell and measuring the current flowing through it. The response to this sinusoidal potential of excitation is an alternating current signal that may be analyzed as the sum total of all sinusoidal functions (Fourier's series)

Another concept that must be considered is that of the capacity or capacitance, expressing the ability of a capacitor to store electric charge. This property determines the relationship between the potential difference existing between the plates of the capacitor, and the electrical charge stored in it, through the following equation.

$$C = \frac{V}{Q} \qquad (2)$$

Where:

C is the capacitance, measured in farads, a relatively big unit that is usually used in submultiples such as microfarads and picofarads.

V is the potential difference measured in volts.

Q is the stored electrical charge measured in coulombs.

On the other hand, conductance is directly related to the ease offered by any material for the flow of the electric current. Conductance G, and resistance are inversely related. The value of the conductance of a material is described in siemens and it is identified by the letter S. One siemens equals 1/W, or also ohm^{-1}.

An increasing application is that of automatically recording impedance in microbiology. Impedance microbiology (IM) is formed by two important techniques: Classical IM, based on the measurement of bipolar impedance or the resistance of the medium (disregarding the dispersion phenomenon) and impedance spectroscopy, also called dielectric spectroscopy basing its measurement specifically on the dispersion of the medium.

IM is the recording of the variability of impedance with time during the growth of the microorganisms in a sample. It is done by placing an arrangement of metal electrodes, submerged in an inoculated culture medium (Figure 1A), thus achieving measurement between the electrodes (bipolar impedance) while the microorganisms found there grow. The technique consists of making measurements of impedance components such as: conductance, capacity, impedance module, phase angle and others through bipolar or tetra polar methods, with electrodes placed in a flask containing an inoculated culture media kept at a constant temperature. These measurements make it possible to record detect, quantify and even identify certain microorganisms found in the samples coming from the industry or from clinical practice

*B*ases of impedance microbiology

In IM, impedance changes are typically measured by the use of a pair of electrodes placed within a growth medium or reacting solution (Figure 1A).

The measurement can be made in two ways: directly, or indirectly. In the direct technique, a pair of metal electrodes is introduced in the medium inoculated with the bacteria we wish to measure. Th metabolic products created during the growth of microorganisms modify the composition of the medium, thus changing the ionic content, which in turn produces a change in the conductivity of the culture media. These changes are recorded through time when variations in the electrode-electrolyte-sample interface are produced. These modifications are proportional to the concentration of living microorganisms that can be recorded through impedance measuring techniques.

The release of the ion by the bacteria into its growth medium (Figure 2) is due to two main mechanisms [3]. The first mechanism is related to the energy metabolism (catabolism) in which, the bacteria consumes oxygen and carbohydrates, and produces carbon dioxide and organic acids. Some simple examples indicate that the conversion of a non-ionized glucose substrate into two molecules of lactic acid would increase culture media conductivity. Furthermore, the metabolism will take the lactic acid and three molecules of oxygen to produce carbonic acid. The smaller and most mobile bicarbonate ion is a more effective ionic conductor than the lactate ion. Hydrogen ions are almost seven times more

3. Owicki J, Parce J. Biosensors based on the energy metabolism of living cells: the physical chemistry and cell biology of extracellular acidification. Biosens Bioelectron 1992;7:257-72.
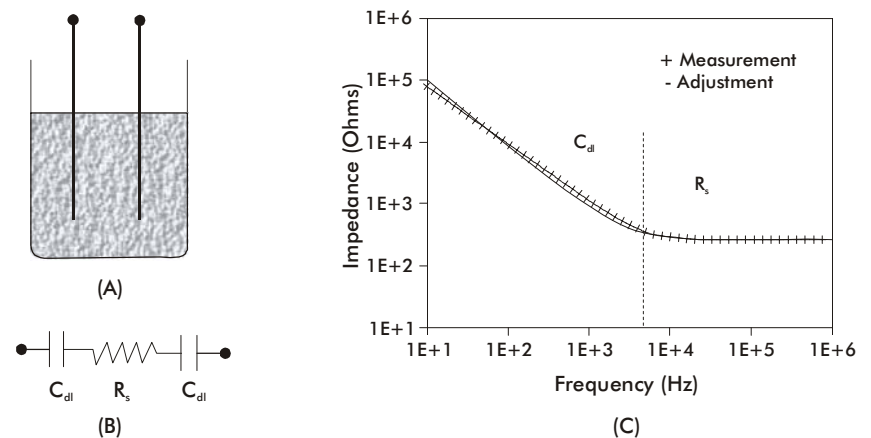

Figure 1. (A) Typical configuration of two electrodes for impedance measurement (B) An equivalent circuit simplified for a two electrode system. (C) Impedance curve vs. frequency.

effective as ionic conductors than as sodium ions, all of which leads to the variation of the impedance caused by this action produced by the bacteria [2]. The second mechanism is related to ion exchange through the cell membrane (active transportation). Ions such as sodium and potassium are actively transported through the ionic channels of the cell membrane (double lipid layer), which function as regulators of the membrane potential and of the osmotic pressure exerted between the inner and outer parts of cells.

Although energy metabolism is the main cause of the release of ions from the cell into its environment; the ionic exchange process also offers a small contribution. What has definitely been clarified is that these ion release processes produce changes in the ionic composition of the culture medium and in its conductivity, which are the bases for the measurement of impedance changes.

In contrast to that of the direct technique, the indirect technique does not measure the changes in impedance directly in the bacterial growth medium. The electrodes, instead of being submerged in the inoculated growth medium, they are introduced into a separate solution (usually into a potassium hydroxide solution). The gases produced by bacterial metabolism (mainly CO_2) are absorbed by the potassium hydroxide solution, producing a decrease in the conductance of the alkaline solution.

In order to detect bacteria, impedance systems measure absolute or relative changes in conductance, capacitance or impedance at regular time intervals during bacterial growth under controlled temperature and humidity. The electric signals measured are graphically represented (amplitude at the vertical axis vs. incubation time at the horizontal axis) and impedance variability curves are generated.

Figure 3 shows the typical impedance curve where it is observed that the impedance is quite stable at the initial part of the curve and then it starts decreasing. The time at which the impedance decreases and crosses a certain threshold value is defined as detection time (t_d). Generally, detection time does not appear until the number of bacteria is of about 106-107 colony forming units (c.f.u.) per mL. When impedance finally reaches its limit, bacteria are at a high concentration of about 108 c.f.u./mL or more, and all the resources in the medium have been metabolized and converted into final products. The shape of the impedance curve corresponds well with the three typical phases of bacterial growth: Phase I, where the bacteria metabolize but do not replicate; Phase II of logarithmic or exponential growth, where the bacteria exponentially replicate, and Phase III, a steady stage, where cell concentration remains relatively constant [4].

A simple theoretical analysis, confirmed by experimental observation, shows that detection time (td, the time needed for impedance to cross an arbitrary threshold), is related to the initial cell concentration (C_o) according to the model represented [5]:

In this expression, a ($t > 0$) and b are constants that depend on the particular type of microorganism, its growth conditions, etc. Eden et al [2] obtained experimental values of these constants: a = 0.96 and b = 7.75, for *td* in hours, and C_o in cfu/mL in the incubation medium.
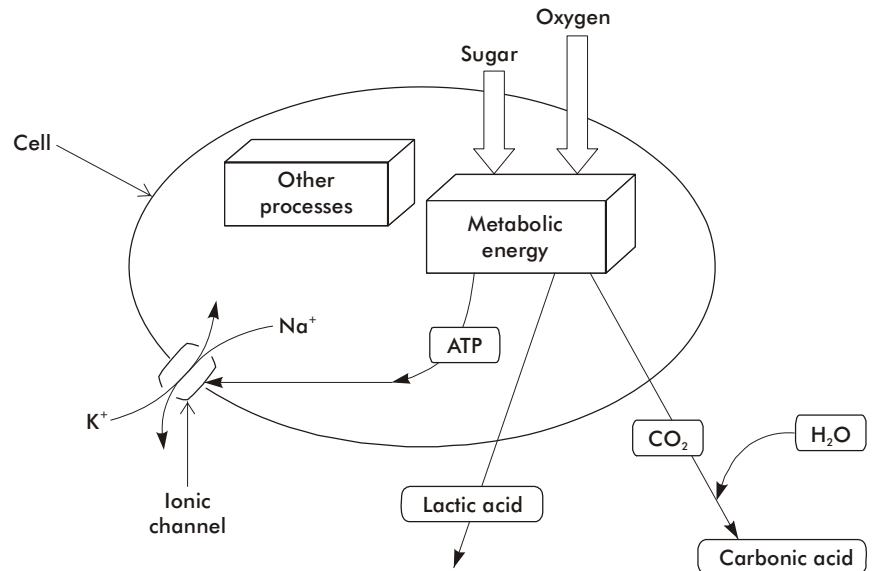

Figure 2. Diagram representing the two main mechanisms responsible for the release of ionic species by living cells: energy metabolism (catabolism) and ion exchange through the cell membrane.
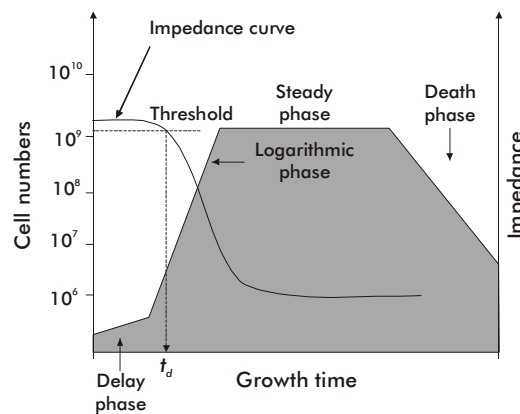

Figure 3. Impedance growth curve with the threshold and detection time together with the two typical phases of bacterial growth.

Figure 4 shows the representation of model (3) for the previous constant values, which indicates that detection time intervals go from 1 hr when C_o is approximately equal to 10^7 c.f.u./mL, up to eight hours when C_o is of approximately 1 c.f.u./mL. Colony forming units is the minimal number of separable cells on the surface (or within it) in a semisolid agar medium giving rise to the development of a visible colony in the order of tenths of millions of progeny cells.

Classical impedance microbiology: culture medium and *salmonella* detection

Many studies have been made to optimize the development of culture media since the direct IM technique is based on the observation of impedance changes. The principles for the design of the culture medium, which are so important for traditional microbiology, are also important for IM. Firstly, the medium must be chosen according to the bacterial growth

4. Talaro KP. Foundations in microbiology. 5th ed. New York: McGraw-Hill. 2005.

5. Gómez R, Bashir R, Bhunia A. Microscale electronic detection of bacterial metabolism. Sensor Actuators B. 2002;86: 198-208.
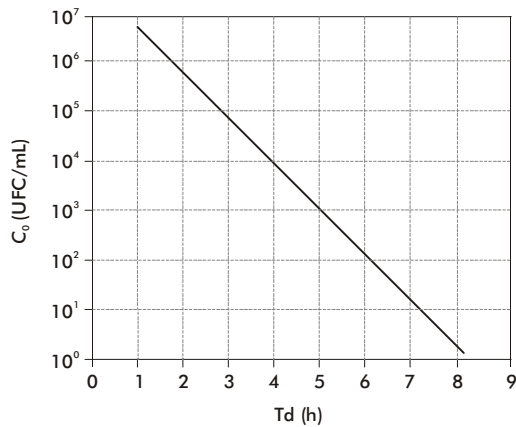
Figure 4. Relationship between detection time and initial cell concentration.

to be analyzed, which grants selectivity to impedance microbiological methods. Secondly, the formulation of the medium should be such that an optimum variability of impedance can be achieved.

Salmonella is the main cause of food intoxication with the highest number of cases reported. Salmonelosis is the infection produced by this bacterium. According to the Center for Disease Control (CDC), salmonelosis is responsible for approximately 1.4 million food intoxication cases more than 600 deaths in the United States each year. These infections produce direct and indirect medical expenses of a billion US dollars per year [6]. Conventional microbiology methods for the detection of *Salmonella* ssp. require three to four days for a presumptive result, and five to seven for its confirmation. Considering the above data, the detection of *Salmonella* spp. has been one of the main concerns of IM studies.

*I*mpedance components

While a great part of microbiological impedance methods only measure the conductance of the medium at a fixed frequency using a pair of electrodes placed within an inoculated medium, several studies have found that total impedance during bacterial growth is composed of two elements that can be measured at different frequency intervals: one refers to the medium, which is called medium or electrolyte impedance, and the other is attributed to the electrode-electrolyte interface, called electrode or interface impedance [7].

*E*quivalent circuit for impedance components

The impedance of the medium and the electrode, as well as their contributions to total impedance, depending on the frequency used, can be properly interpreted by means of an equivalent circuit of the system.

For these elements of the equivalent circuit to be useful, they must always be based on the electrochemical physics of the system. Basically, the impedance between two electrodes (Figure 1A) may be represented by a simple circuit connected in series as shown in figure 1B, formed by the resistance of the solution between both electrodes (R_s) and the capacitors of the metal-sample interface (one for each electrode: C_{dl}).

Yang *et al.* in 2003 [8] demonstrated the feasibility of using an equivalent circuit to analyze the impedance detection system for bacterial growth. They showed that the impedance spectrum obtained in a growth medium with 1.1 x 103 c.f.u./mL of *Salmonella typhimurium* corresponded to the adjusted spectrum (shown in figure 1C), which corroborates the validity of the equivalent circuit used to justify impedance changes in the system.

Based on the equivalent circuit, when a sinusoidal potential of alternating current is applied to the system, the impedance (z) of the section between the electrodes is a function of its resistance (R_s), its capacitance (C_{dl}), and also of the applied frequency (f), as expressed in equation 4:

$$/z/ = \sqrt{(R_s)^2 + \left(\frac{1}{C_{dl}}\right)^2} \qquad (4)$$
$$\pi f$$

The above model explains the impedance variability curve (Figure 3), where it always decreases when the concentration of bacteria grows in the culture medium. The decrease in impedance has two causes: the decrease of R_s, and the increase of C_{dl}. It is acknowledged that bacteria metabolize uncharged large molecules producing small charged molecules, thereby decreasing the resistance of the medium (R_s). The increase of the capacitance of the electrode-sample interface is related to the change in the ionic composition of the medium in the area surrounding the metal surface, which strengthens the formation of a double layer. The value of the capacitance depends on many factors, which include the electrode potential, temperature, the ionic concentration of the medium, the types of ions, and the properties of the electrode surface (electrode rugosity, absorption, etc). In this case, the capacitance at the double layer thus formed, may be expressed as follows:

$$C_{dl} = \frac{A\varepsilon_{dl}}{d} \qquad (5)$$

Where:

ε_{dl} is the dielectric permittivity at the electrically charged double layer; $\varepsilon_{dl} = \varepsilon_0\,\varepsilon_p$, ε_0 is the permittivity of the open space, and ep is the effective dielectric constant of the layer separating the ionic charges of the electrodes; A is the electrode area, and d is the thickness of the double layer.

Before bacterial growth, the medium contains uncharged or weakly charged substrates such as lactose. During growth, these compounds are transformed into highly electrically charged small ions. As a result, the number of polar molecules and of charged small molecules at the double layer increases, thereby increasing the dielectric permittivity edl, and at the same time reducing the double layer thickness (*d*). These combined changes provoke an increase in capacitance, and as a result, the impedance decreases.

Expression (4) also gives the best possible explanation on the properties of the impedance measurement during bacterial growth, which also depends on the frequency. As shown in figure 1c, total impedance decreases after the increase of the frequency in the low frequency interval, from 10Hz to 10kHz, while

6. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C *et al.* Food-related illness and death in the United States. Emerg Infect Dis 1999;5:607-25.

7. Felice CL, Valentinuzzi ME. Medium and interface components in impedance microbiology. IEEE Trans Biomed Eng. 1999;46:483-7.

the impedance becomes independent from the frequency at the high frequency interval (between 10 kHZ and 1 MHz). In the low frequency zone (f < 10 kHz), capacitance of the double layer essentially offers high impedance and it turns it into the main source of the total impedance of the system, so that the resistance of the medium can be ignored. This region is defined as the capacitive region of the double layer, at which the electrode impedance can be detected (Figure 1C, C_{dl} region).

On the other hand, at the high frequency interval (f > 10 kHz) there is no substantial contribution to the double layer capacitance. Thus, the most important contribution to the total impedance of the system at high frequencies is related to the resistance of the medium, which is independent from the frequency. This region is defined as the resistive region, in which ion conduction in the medium is dominant (Figure 1C, R_s region). Therefore, the changes in the double layer of the electrode, and the changes in the medium during bacterial growth can be detected by measuring impedance at different frequencies, which is the reason for the present study to develop new systems that may improve the processes of microbiological detection in relation to resolution and time taken for diagnosis.

*H*istorical background and applications in biotechnology

It can be considered that the last decade of the XIX Century marks the beginning of IM. As long ago as 1890, the American researcher GN Stewart started a series of experiments introducing elements of conductance and conductivity as parameters to estimate circulation time and the volume of the heart output [9]. In July 1898, Stewart made a presentation at the British Medical Association in Edinburgh titled "The changes produced by the growth of bacteria in the molecular concentration and the electrical conductivity of culture media", which gave rise to impedance bacteriometry and was published the following year [10]. The growth curves he obtained were very similar to those now obtained with the impedance systems available (Figure 5), with the notable difference in the extraordinary speed of the present systems, while Steward had to work on the measurement for more than 30 days.
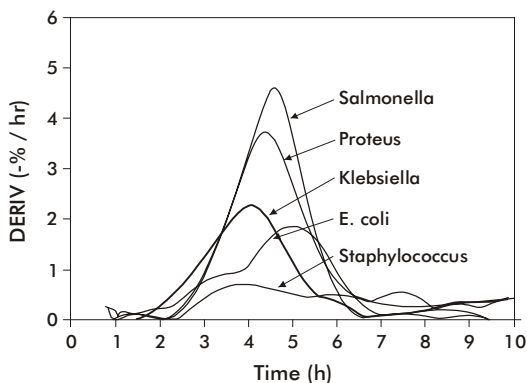
Figure 5. The derivatives of the curves of the impedance module for different bacteria of clinical origin growing in a BHI broth at 37 °C (Felice CJ, PhD Thesis, INSIBIO-UNT, Tucumán, Argentina, 1995).

In 1957, Schwan publishes a very important paper on the electric properties of tissues and cells in suspension [11]; but it was not until the 1970's that IM started to expand, through the increasing number of papers published that greatly promoted it and spread it worldwide. During this period, outstanding papers such as those of Ur [12, 13] and Cady [14, 15] were published. The works of the groups of Eden and Torry in the United States set the bases for the IM, which gave way to the introduction of the Bactometer and Malthus measurement systems, respectively [2, 16].

From 1975 to 1999 new papers were published on this topic especially important were the contributions of Felice and Valentinuzzi [7]. In that period, the papers on the practical applications of the methods were mainly concerned with food industry and dairy products, where it was used as a tool for quality control. Some of these detection and quantification applications were performed in either raw or pasteurized cow milk. Cady *et al.* [17] and Gnan-Luedecke [18], were the first to propose the use of impedance as an alternative method for plate counts

Impedance was also successfully applied in the study and recording of microbial load of a wide range of food products that include: vegetables [19], cereals [20-21], sweets [22], and meat [23]. Moreover, the technique was also used to identify groups of microorganisms among which coliform bacilli in meat [24], gram negative bacteria in pasteurized milk [25], and *Salmonella* [26-28] were included; as well as for the evaluation of antibiotics[29].

Applications for detection and quantification have also included beer [30], wine [31], fish [32], pharmaceuticals, and cosmetics [33], as well as fruit juices [34]. Other applications have dealt with sewage effluents to detect coliform bacteria [35], and for the detection of urinary infections [36], or in the human blood [37]. A less common application has been in the study of antibiograms [2]. In this case, turbidimetry already has equipment and experience available at a commercial scale [38]; but there is still no automated commercial system based on impedance measurement that is able to make antibiograms.

Several commercial analytical instruments are based on the classical IM principle, for instance: the Malthus System (Malthus Instruments, Crawley, West Sussex, UK), Bactometer (bioMerieux, Hazelwood, MO, USA), the rapid automated bacterial impedance technique (RABIT) (Don Whitley Scientific Ltd., Shipley, UK) and BacTrac (Sy-Lab, Purkersdorf, Austria) [29, 39-41], respectively.

Impedance techniques can also be used to monitor the form of bacterial growth. In 1998, Fehlhaber and Kruger found that different species of bacteria, under different conditions, showed specific impedance growth curves [42].

The new interdigitated microelectrodes have radically revolutionized research on impedance spectroscopy (Figure 6A). Microelectrodes have many advantages over conventional electrodes in regard to analytical measurements because of the low resistance and high signal-sound ratio, because they reach the steady state faster and because of the use of small volumes of the solutions [43]. In the last decade, the microelectrodes in the form of interdigitated arrange-

8. Yang L, Ruan C, Li Y. Detection of viable *Salmonella typhimurium* by impedance measurement of electrode capacitance and medium resistance. Biosens Bioelectron 2003;19:495-502.

9. Stewart GN. Researches on circulation time and on the influences which affect it. IV. The output of the heart. J Physiol (London). 1897;XXII:25.

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25. Nieuwenhof FFJ, Hoolwerf JD. Detection of post-pasteurization contamination of pasteurized milk with the Bioscreen, Nederlands Instituut voor Zuivelonderzoek, NIZO-EDE Report. 1205. Nov 1986:10.
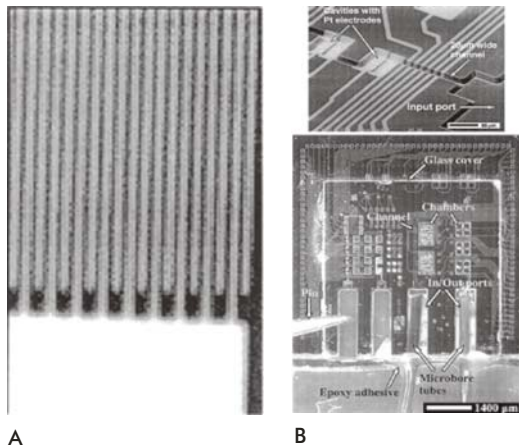
A   B

Figure 6. Electron microscopy zoom of an interdigitated microelectrode (A) and of the channels of a biochip belonging to a micro fluid device (B).

ments have been of interest in the fields of biosensors and impedimetric immunosensors [44-47].

The trend in current research for the detection of pathogenic elements is focused on the production of biosensors for the measurement of spectra of impedance. These biosensors have been able to detect particular ones and the responses have been obtained much faster.

Applications including the fast determination of *Salmonella typhimurium* have shown the feasibility of using the interdigitated microelectrodes to measure impedance for the purpose of monitoring bacterial growth [48]. The volume of the analyzed sample can be reduced from 10-15 mL to 1-2 mL. Furthermore, detection time for the same initial concentration of bacteria is reduced to 3 or 4 hours when compared with the use of the conventional electrode system (Figure 7).

The miniaturization of impedance systems known as *biochip* or *lab-on-a-chip* to detect bacteria has increased the hope of reaching a faster detection of bacterial growth (Figure 6B). Gomez et al were among the first to make this type of device to detect impedance changes caused by microbial metabolism. The basic idea is to confine a few living cells into a volume in an
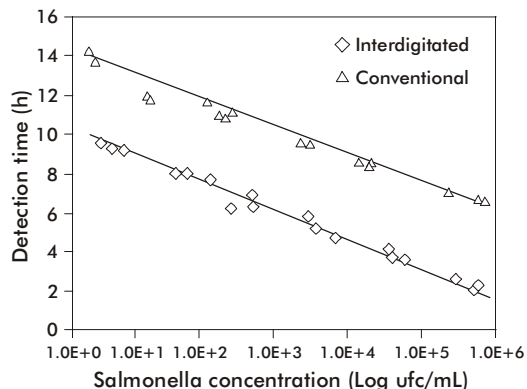

Figure 7. Comparison of detection time of bacterial growth using an interdigitated microelectrode system and the conventional electrode system.

order starting from nanoliters to picoliters, so that the few living cells in a low conductivity buffer solution may be rapidly detected measuring impedance using interdigitated microelectrodes. Other studies focus the application of this new method known as IM dielectrophoresis (DEPIM), combined with electropermeabilization using a chip as the support. Dielectrophoresis (DEP) is the electrokinetic movement of dielectrically polarized particles in a "non uniform" electric field [50]. Progress in the development of microelectrode arrangements has made DEP a highly useful technique for manipulating biological cells in micro-fluid devices, biochips, and biosensors. With this method 10^2 c.f.u./mL were detected in 3 hours

Most of the IM applications have been widely reviewed by relevant researchers [54-60].

*F*uture goals

Taking into account the results obtained in the detection of pathogenic microorganisms when combining impedance spectroscopy with new techniques for cell manipulation, and the use of reduced volumes, it is considered that the most important restriction for the use of this technique is that it is very young. Advances in the micro-manufacturing of devices and biochips, that can store volumes in the order of the nanoliters and picoliters where very few bacteria are confined would be possible, but their development would require at least a decade. Another problem would consist in the mass production of these devices preventing the exclusiveness of its use only for the elite. An increase in the sensitivity of this technique is feasible with the use of biosensors based on new geometric models of interdigitated electrodes, together with a change in mathematical data processing.

*C*onclusions

As observed, IM and its applications in the detection of pathogenic microorganisms, together with the current use of interdigitated microelectrodes, the development of miniaturization, and the integration of biosensors with other techniques such as dielectrophoresis and electropermeabilization will surely lead to future developments. The main purpose of this integration is to increase the sensitivity of detection through the use of a reduced volume of the sample. Other aspects that must be implemented are the need of redefining the theoretical bases, the development of new components, the principles for designing the culture media and the use of equivalent circuits for the analysis of impedance systems.

The impedance technique as a transduction principle has become a promising field for the development of rapid and effective methods to detect microbiological growth. In spite of the fact that IM was established more than 100 years ago, it is just now entering into a new stage based on miniaturized devices (nanosciences and nanoelectronics). Advances in micromanufacturing have brought about the conditions for the development of micro-devices and biochips, which have proven their efficacy in maximizing the impedance signal, increasing sensitivity and reducing time in the detection of pathogenic microorganisms. This trend confirms that among the new automated methods those of impedance microbiology are the most successful.

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