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Electronic tongue for simultaneous detection of endotoxins and other contaminants of microbiological origin

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ABSTRACT

Endotoxins, also referred to as pyrogens, are lipopolysaccharides (LPS) present in the outer membrane of Gram-negative bacteria, and represent one of the most dangerous microbiological contaminants in water for hemodialysis and intravenous infusion. A method is presented for the simultaneous detection of endotoxins and other bacterial lysis contaminating species in purified water for parenteral formulations. The technique used is electrochemical impedance spectroscopy, with data interpretation using principal component analysis (PCA), cluster analysis (CA), and multivariate discriminant analysis (MDA). Two types of electrode surfaces were modified with LPS recognition agents: (i) a 37 amino acids fragment of a 18 kDa cationic antimicrobial protein (CAP18F) that has LPS binding activity; (ii) the highly selective endotoxin neutralizing protein (ENP). Statistical multivariate analysis of the impedance spectral data allowed the detection of endotoxin at, and below, the threshold pharmaceutical regulatory level. Discrimination of LPS from samples containing proteins, nucleic acids, phospholipids or their mixtures was achieved. These results open a new route to a practical instrumental method capable of detecting and discriminating LPS from other potential pro-inflammatory species of microbiological origin, such as nucleic acids.

1. Introduction

Microbiological water quality is extremely important in medical treatments where aqueous solutions have access to the bloodstream, such as intravenous infusions and hemodialysis solutions. In hemodialysis therapy, patients with renal failure are not only exposed to higher volumes of water as compared with the general population, but also the barrier between blood and dialysis fluid is a semipermeable membrane, allowing the access of several contaminants into the bloodstream (Hoenich and Levin, 2003). These membranes are very effective in avoiding the passage of whole microorganisms, however their efficiency is limited regarding many relatively small sized toxic or potentially pro-inflammatory products coming from bacterial lysis (*e.g.*: lipopolysaccharides, DNA, proteins, and others).

Two tests are ordinarily used to assess microbiological contamination in fluids: direct bacterial counting via specific cultures (number of colony forming units), and the Limulus Amebocyte Lysate (LAL)-test detecting the presence of pyrogenic lipopolysaccharides (LPS) coming from the lysis of Gram-negative bacteria. The CFU test has the drawback that many microorganisms that survive in highly purified water require very specific culture conditions. On the other hand, water purification modules utilizing membrane filters rule out the presence of bacteria in the product, but are not able to filter off many bacterial decomposition products, such as LPS endotoxin. The LAL test detects presence and concentrations of intact LPS, and by extension (in the absence of other straightforward tests) is taken as a global indicator signaling the presence (or assuring the absence) of pyrogens and/or pro-inflammatory species (Schindler, 2009).

Lipopolysaccharides are present in the outer membrane of all Gram-negative bacteria. Although LPS itself is chemically inert, its presence in blood (endotoxemia) sets off a cascade of host responses affecting the structure and function of organs and cells, changing metabolic functions, raising body temperature, modifying hemodynamics, and causing septic shock (Van Amersfoort et al., 2003).

In the last 20 years a great improvement has been made in the study of chemical interactions between lipopolysaccharides and antibacterial proteins. Some of the systems that have been studied are as follows: endotoxin neutralizing protein (ENP), produced by horseshoe crab (Hoess et al., 1993), cationic antibacterial proteins from humans (Larrick et al., 1995), peptides derived from sequences of the above-mentioned proteins (Ried et al., 1996); and polymyxin produced by *Bacillus polymyxia* (Thomas et al., 1998). These find-

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ings allow the development of an assay equivalent to those based upon antigen–antibody interaction, and some examples can be found in the recent years (Priano and Battaglini, 2005; Priano et al., 2007; Pallarola and Battaglini, 2009; Voss et al., 2007; Jones and Jiang, 2005). In all the cases, labeling of the endotoxin (Priano and Battaglini, 2005; Priano et al., 2007, Pallarola and Battaglini, 2009) or the recognition agent (Voss et al., 2007; Jones and Jiang, 2005) is needed.

Studies of the chemical structure of pyrogenic LPS present in cell walls of Gram-negative bacteria (Scheme S1, supplementary data), have shown that lipid A is the biologically active moiety responsible for pyrogenic effects (Takahashi et al., 1987), composed of a hydrophilic, negatively charged bisphosphorylated diglucosamine backbone and a hydrophobic domain of six (*Escherichia coli*) or seven (*Salmonella*) acyl chains in amide and ester linkages (Rietschel et al., 1993; Zahringer et al., 1994; Taylor et al., 1995). This molecule is structurally able to interact with suitably modified surfaces, such as via electrostatic and hydrophobic interactions, coordination through the phosphate groups or, more selectively, binding to a recognizing protein as those previously mentioned.

Despite the widespread acceptance of the LAL test for controlling the absence of pyrogenic and pro-inflammatory molecules in aqueous systems, there is an increasing concern, suggesting that the accepted regulatory tests may not detect all bacterial decomposition products with possible detrimental effects on patients (Schindler, 2009). Therefore, a simple instrumental method, able to rapidly assure the absence of LPS above a very low threshold value, and/or trigger an alarm if detecting the presence of LPS, even in the presence of minute amounts of other contaminants of microbiological origin, would be very convenient.

Considering that the source of LPS in the samples of our interest is Gram-negative cell material, it is expected that other species of bacterial debris will also be present together with LPS, superimposing their response in all assays. The composition of Gram-negative bacteria comprises mainly water (70%), proteins (15%), nucleic acids (7%) and to a lesser extent lipids and polysaccharides (Nelson and Cox, 2000); these species, or their fragments, will constitute the probable coexisting impurities.

Impedance spectroscopy is an electrochemical technique able to provide very rich information, due to its ability to rapidly scan the experimental system at different frequencies, generating a large set of data. Depending on the frequency, the response comprises information arising from interactions of the components of the sample with the electrode surface, affecting properties such as the capacity, the electron transfer processes, and conductivity of the system. Suitable structural modifications of electrode surfaces, introducing affinity sites enhancing analyte to surface interactions, should favor such effects. Although this amount of information cannot be described by simple models, a suitable statistical data analysis may allow to differentiate and discriminate the composition of different samples (Pioggia et al., 2007a,b; Ferreira et al., 2003; Riul et al., 2003; Muñoz-Berbel et al., 2008).

In this work, we demonstrate that electrochemical impedance spectroscopy combined with PCA on specially modified electrodes for LPS recognition allows discriminating LPS, proteins, nucleic acids and phospholipids in very low concentration mixtures, both in purified water or physiologic saline solutions. At the same time, the technique permits to detect LPS at concentrations as low as 10 pg mL^{-1}. In one case, the electrode surface was modified with a 37 amino acids fragment of an 18 kDa cationic antimicrobial protein (CAP18F) that has LPS binding activity (Larrick et al., 1994), and is able to interact with phosphate groups and the amphiphilic moiety present in the endotoxins (lipid A). In another approach, the electrodes were modified with endotoxin neutralizing protein (ENP) (Hoess et al., 1993), a protein with very specific LPS binding capacity. A signal array, constructed from the impedance spectra of each electrode, was used in a multivariate data analysis, allowing not only the detection of endotoxins at regulatory levels (United States Pharmacopoeia 30, 2007; Nystrand, 2008), but also the discrimination of other types of microbiological contaminating species that may be present, and their mixtures.

2. Materials and methods

2.1. Materials

Smooth-form lipopolysaccharide from *Salmonella enterica* sv. Minnesota was supplied by Sigma–Aldrich Argentina. LPS purification is described elsewhere (Pallarola and Battaglini, 2008). LPS is a pyrogen. It may cause fever. It may be harmful by inhalation, ingestion or skin absorption. Good laboratory technique should be employed: wear lab coat, gloves and safety glasses. Work in a well ventilated area. Avoid contact with open wounds. Aniline, 3-aminophenylacetic acid (3-AFA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), N,N-bis(carboxymethyl)-L-lysine (dNTA), bovine serum albumin (BSA), deoxyribonucleic acid sodium salt from salmon testes (DNA) and 1,2-dipalmitoyl-sn-glycero-3-phosphate disodium salt (DPGP) were provided by Sigma–Aldrich Argentina. Details of Endotoxin neutralizing protein (ENP), CAP18F and LAL test are given in Supplementary material. Apyrogen water was provided by Lonza (<0.005 EU mL^{-1}). Ultrapure water (resistivity: 18 MΩ cm) was obtained with a Millipore MilliQ water purification system. Physiological saline solution was provided by Parafarm (Argentina). All the other reagents were analytical grade.

2.2. Sensor construction

2.2.1. Ni-dNTA sensor

A disposable 8 graphite electrode array was constructed by screen printing as previously reported (without silver underlying tracks) (Priano et al., 2008). Sequential polymerization of aniline and 3-AFA was carried out by cyclic voltammetry between −0.2 and 0.85 V vs. Ag/AgCl at 10 mV s^{-1}. First, the electrodes were immersed in a solution of 90 mM aniline in 1.8 M H_2SO_4 and the potential was simultaneously cycled 6 times; then, the electrodes were rinsed with water, and immersed in a solution of 10 mM 3-AFA in 1.8 M H_2SO_4 and the potential cycled 4 times. Finally, the electrodes were rinsed with ultrapure water. The carboxylic groups present in the electrodes were activated with a solution containing 50 mM EDC, 125 mM NHS in MES buffer, pH 5.5 for 30 min. After the activation step, the electrodes were rinsed with MES buffer, pH 5.5 and immersed in a 5 mM dNTA in 50 mM PIPES buffer, pH 8.0 for 1 h. Then, the electrodes were rinsed with ultrapure water and immersed in 1 mM sodium hydroxide for 10 min to deprotonate the carboxylic groups for further Ni complexation. The electrodes were rinsed with ultrapure water again and immersed in a 50 mM $NiCl_2$ solution for 1 h. Finally, the electrodes were rinsed with ultrapure water. Each electrode was divided in two by making an incision with a scalpel, leaving an insulating gap of 40 μm between them (Scheme S2). One half was used as working electrode and the other as counter electrode. The 8 sensors were separated and the samples were individually analyzed.

2.2.2. CAP18F sensor

The construction was similar than before. After the Ni-dNTA electrode preparation, the system was exposed to 1 μM CAP18F solution in 20 mM Tris buffer (pH 7.4, 20 mM NaCl) for 1 h. Then, the electrodes were rinsed with ultrapure water.

2.2.3. ENP sensor

Aniline and 3-AFA were sequentially electropolymerized and the carboxylic groups activated as for Ni-dNTA sensors. Then, the electrodes were exposed to 1 μM ENP in 50 mM PIPES buffer, pH 8.0, for 1 h. Afterwards, the electrodes were rinsed with ultrapure water. Each electrode was divided in two as before to produce a working and a counter electrode separated by a 40 μm gap.

For all the sensors, the amperometric response of the electrode was stabilized by cycling the electrode at 10 mV s^{-1} between −0.2 and 0.55 V 10 times, and then left at 0.2 V vs. Ag/AgCl for 200 s.

2.3. Electrochemical impedance spectroscopy (EIS) measurements

EIS measurements were performed using a μAUTOLAB type III impedance analyzer. The cell was made in acrylic, the reference electrode was placed over the working and counter electrodes fixed at a distance of 70 μm (Scheme S2B). The impedance spectra were recorded within a frequency range of 0.1 Hz–10 kHz. The potential was fixed at 0.2 V for all the sensors. The amplitude of the alternating voltage was 10 mV. A total of 51 frequencies were studied with logarithmic distribution in each decade. Concentration of the tested samples were: LPS ranging from 0.01 to 10 ng mL^{-1}, DNA from 0.01 to 1 ng mL^{-1}, DPGP from 0.7 to 70 μg mL^{-1} (0.1–10 μM), and BSA from 5 to 500 μg mL^{-1}. Mixtures were prepared using 0.1 ng mL^{-1} LPS plus one of the contaminant in a concentration ratio contaminant: LPS equal to 100, 10, and 1. Each sample was tested in a new modified electrode, where first apyrogen water or physiological solution was analyzed, and then the respective sample. All samples were incubated for 15 min. Results were analyzed in terms of resistance (Z') and capacitive reactance (Z'').

2.4. Multivariate data analysis

The multidimensional response pattern was evaluated statistically for different sensors in the presence of microbiological contaminants (LPS, DNA, BSA, DPGP, LPS + DNA, LPS + BSA, LPS + DPGP) using principal component analysis (PCA) (Jolliffe, 2002), cluster analysis (CA) and multivariate discriminant analysis (MDA) (Johnson and Wichern, 2002; Scott et al., 2006). For the case of one sensor 102 dimensions were analyzed, whereas in the case of two sensors 204 dimensions. Details regarding the statistical methods applied in this work can be found in Supplementary material.

3. Results and discussion

3.1. Sensor construction and impedance response

Considering that apyrogen water is a matrix of very low conductivity, an electrode array was developed to perform experiments in these conditions; the working and the counter electrodes are two parallel graphite strips at a distance of 40 μm, while the reference electrode is placed onto them at 70 μm (details can be found in Scheme S2B). This arrangement provides a compact and reproducible system in which the ohmic drop due to the low water conductivity, representing a small part the information contained in the impedimetric signal, can be handled by means of a conventional potentiostat.

A reactive layer was deposited on the graphite electrodes, enabling further chemical modifications of it in order to generate suitable discriminating affinities towards the analytes. First, electropolymerization with a thin layer of polyaniline (PANI) was effected, followed by another application onto the former polymer with 3-aminophenylacetic acid (3-AFA). In this way, not only acetic groups are anchored to the surface for further modification,

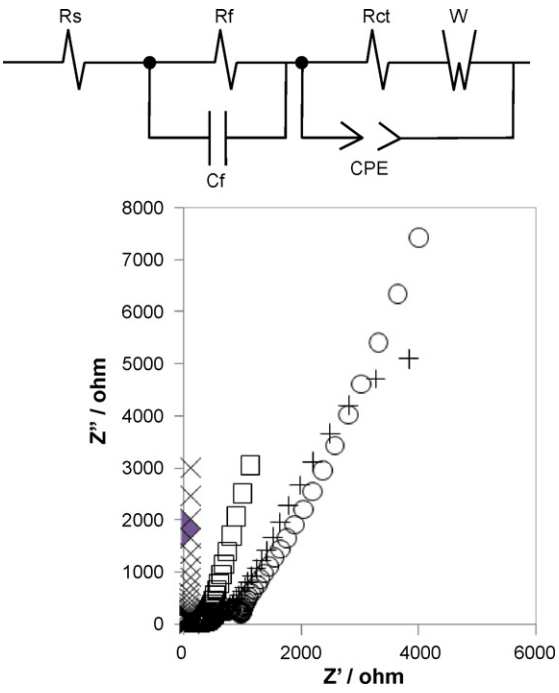

Fig. 1. Top: Proposed equivalent circuit for the observed impedance response: Rs, solution resistance; Cf, capacitance of the polymer film; Rf, polymer film resistance; CPE, constant phase element; Rct, charge transfer resistance; and W, finite length Warburg impedance. Bottom: Nyquist plot for CAP18F sensors exposed to different samples, (×) apyrogen water; (□) 0.01 ng mL^{-1} LPS; (+) 0.7 μg mL^{-1} DPGP; (○) 5 μg mL^{-1} BSA. The relative standard deviations ($n = 3$) for all the samples is less than 4%.

but also the PANI, at neutral pH, remains electroactive, generating a redox probe that can provide additional useful information, as reported for PANI modified with N-(3-propylsulfonic acid) aniline (Yánez-Heras et al., 2007).

As described in Section 2, graphite electrodes were built by screen printing techniques in plates containing eight electrodes, and later modified simultaneously to obtain a better reproducibility. Once modified, each plate was exposed to one of the matrixes used in this work. EIS experiments were carried out applying a 10 mV signal at 51 frequencies from 10^{-1} to 10^4 Hz. The average data obtained from the eight electrodes can be informed as resistance (Z') and capacitive reactance ($Z'' = 1/\omega C$); As an example, for eight ENP modified electrodes exposed to apyrogen water, the average values for the resistance and the capacitive reactance at 10 Hz are 305 ± 2 and 114 ± 2 Ω, respectively ($n = 8$). A Nyquist plot, taking into account all the frequencies, is shown in Fig. S1. The fabrication reproducibility permits correlate results, without the need of relating them to a common reference; a similar behavior is observed for the other sensors.

Recently, other research groups have used electroactive polymers in impedance based immunosensors (Hafaida et al., 2010; Giroud et al., 2009). In these works the impedance spectra correspond to the typical behavior observed for electroactive films, proposing an equivalent circuit as the one depicted in Fig. 1; their results show a great similarity to our spectra. Thus, Fig. 1 shows the results obtained using one of the electrodes (the sensor modified with CAP18F) for apyrogen water, and for a set of samples containing different solutes (the pertinent parameters are identified in the caption). In the case of apyrogen water the film acts as a capacitor in series since, due to the lack of ions, the film presents a high resistance (Rf). Once the film is exposed to solutions containing LPS or other compounds, important changes are observed attributed to interactions of the added species with the polymer film. The interpretation of the nature of these interactions is not simple; the most

evident changes are observed at low frequencies, and they may be assumed to be due to the adherence of charged compounds onto the modified surface of the electrode affecting the resistance and capacity of the film.

Our focus was placed in using the wealth of information of the impedance scan data measured with the sensors, for searching differences that would allow determining the presence of different possible residues of bacterial cells, in concentrations within very low threshold figures. To this purpose, a multivariate treatment of data was attempted (PCA, CA and MDA), exploring also the ability of the present methodology to discriminate between different compositions.

3.2. Common experimental conditions

Two types of matrixes were tested, apyrogen water and physiological saline solution. Since the main components of Gram-negative bacteria are proteins and nucleic acids, bovine serum albumin (BSA), DNA, and 1,2-dipalmitoyl-sn-glycero-3-phosphate disodium salt (DPGP) (a phospholipid with chemical similarities to LPS), were used as model contaminants in order to investigate the effect of possible interferences by eventual contaminating species.

The LPS concentrations tested were in the range of 0.01–10 ng mL^{-1}. The lowest dilution of endotoxic activity was 0.03 EU mL^{-1}, equal to the lower limit assured by the conventional LAL test. The concentrations of the other compounds are listed in Section 2.3.

Impedance spectroscopy was carried out using 51 frequencies within the range 10^{-1} to 10^{4} Hz, rendering a current, and a phase shift response, for each frequency. The results can be represented as resistance (Z') and capacitive reactance (Z''); therefore, for each sensor and for each sample, 102 bits of information are available for PCA.

3.3. Ni-dNTA sensor

The first sensor tested was an electrode derivatized with a Ni-dNTA complex (Scheme S2C), similar to the one used in immobilized metal ion affinity chromatography (IMAC). This type of chromatographic separation is based on the interactions of histidine tags present in proteins with the Ni complex. However, Ni moieties can also interact with phosphate groups (Gobi and Ohsaka, 2000). In our case, since three of the four substances included in our samples contain phosphate groups (structures are represented in Scheme S1), we expected that interactions between the Ni moieties and phosphate groups would show up in the spectra as useful differentiations of electrical behavior.

After running the impedance spectra, LPS detection was carried out analyzing the responses for each sample by PCA. PCA reduces the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. This is achieved by transforming to a new set of variables, the principal components (PCs), which are uncorrelated, and which are ordered so that the first few retain most of the variation present in all of the original variables (Jolliffe, 2002). The first principal component contains the highest degree of variance and other components follow in the order of decreasing variance, in this way the pattern generated by the sensor is reduced to a single score and plotted in the new generated space using only two or three principal components; the PCA concentrates the most significant characteristics (variance) of the data into a reduced dimensionality space.

This representation (score plot) is shown in Fig. 2A and B. The PCA score plot utilizes the first two principal components; in each axis the percentages indicate the participation of each dimension in the total variance observed in the matrix. It is particularly noteworthy that in both cases the first two PC account for more than 90% of the total variance. In apyrogen water (Fig. 2A), the sensor is able to discriminate BSA, the pure matrix and the samples containing phosphate are randomly dispersed. While for the physiological saline solution, the PCA shows a good discrimination among the pure solution, the protein contaminated solution and samples with substances containing phosphate moieties (Fig. 2B). From these results, the use of Ni-dNTA modified electrode as a sensor shows a limited applicability if an effective discrimination of endotoxins is required.

3.4. Ni-dNTA/CAP18F sensor

To improve the discrimination of LPS from the other phosphate containing compounds, a peptide able to recognize LPS was bound to the Ni-dNTA complex though a His-tag (Scheme S2C). The peptide chosen is the LPS binding domain corresponding to an 18 kDa cationic antimicrobial protein (CAP18F) (Larrick et al., 1994), which was synthesized plus 6 histidine residues to be bound to the Ni-dNTA complex.

EIS experiments were carried out as above. The PCA graphs obtained from these data are presented in Fig. 2C and D, both for apyrogen water and physiological saline solution, respectively. It can be observed that in both systems BSA is still markedly discriminated, but also each of the phosphate containing compounds shown a characteristic pattern.

Cluster Analysis, CA, was performed in order to quantitatively confirm the visually observed grouping in the PCA graph. In the case of physiological saline solution, all the 5 groups are successfully clusterized. Yet, for the water matrixes, some LPS samples are indistinguishable from pure apyrogen water, while the other three groups are correctly discriminated.

Although, as previously stated, discrimination of different contaminants of microbiological origin is one of the searched targets of interest, the regulatory standard today is specifically focused on the endotoxin threshold value (i.e. 2 EU mL^{-1}, or less, depending on the end use of the purified water) (Nystrand, 2008). Thus, further improvements on specificity of the technique are required to this effect.

3.5. ENP sensor

Seeking a better recognition capacity of LPS, a third sensor was developed, modifying the surface, with endotoxin neutralizing protein (ENP), a specific LPS recognizing molecule. The electrode is basically similar to the second model discussed above; the difference being that phenyl acetic moieties, once activated, are now directly modified with the recognition protein (Scheme S2C).

EIS experiments were carried out as previously described. PCA of the data are presented in Fig. 2E and F, for the apyrogen water, and for the physiological saline solution series, respectively. In the physiological saline matrix series (Fig. 2F) a good discrimination between all the samples is noted, while in the case of the apyrogen water series (Fig. 2E), three main groups can be described: apyrogen water, LPS contaminated samples, and samples contaminated with other compounds. It is noteworthy that this sensor is unable to distinguish the globular BSA, which appears confused with other compounds (DNA and DPGP).

Observing the results obtained for the three different electrodes, the effective selectivity for LPS of the this sensor containing ENP is noted, as well as the ability of the sensors containing exposed Ni centers to discriminate between BSA and compounds containing phosphate groups.

The high selectivity of this third sensor suggests that the ability to work as a quantitative tool is worth investigating. The signal

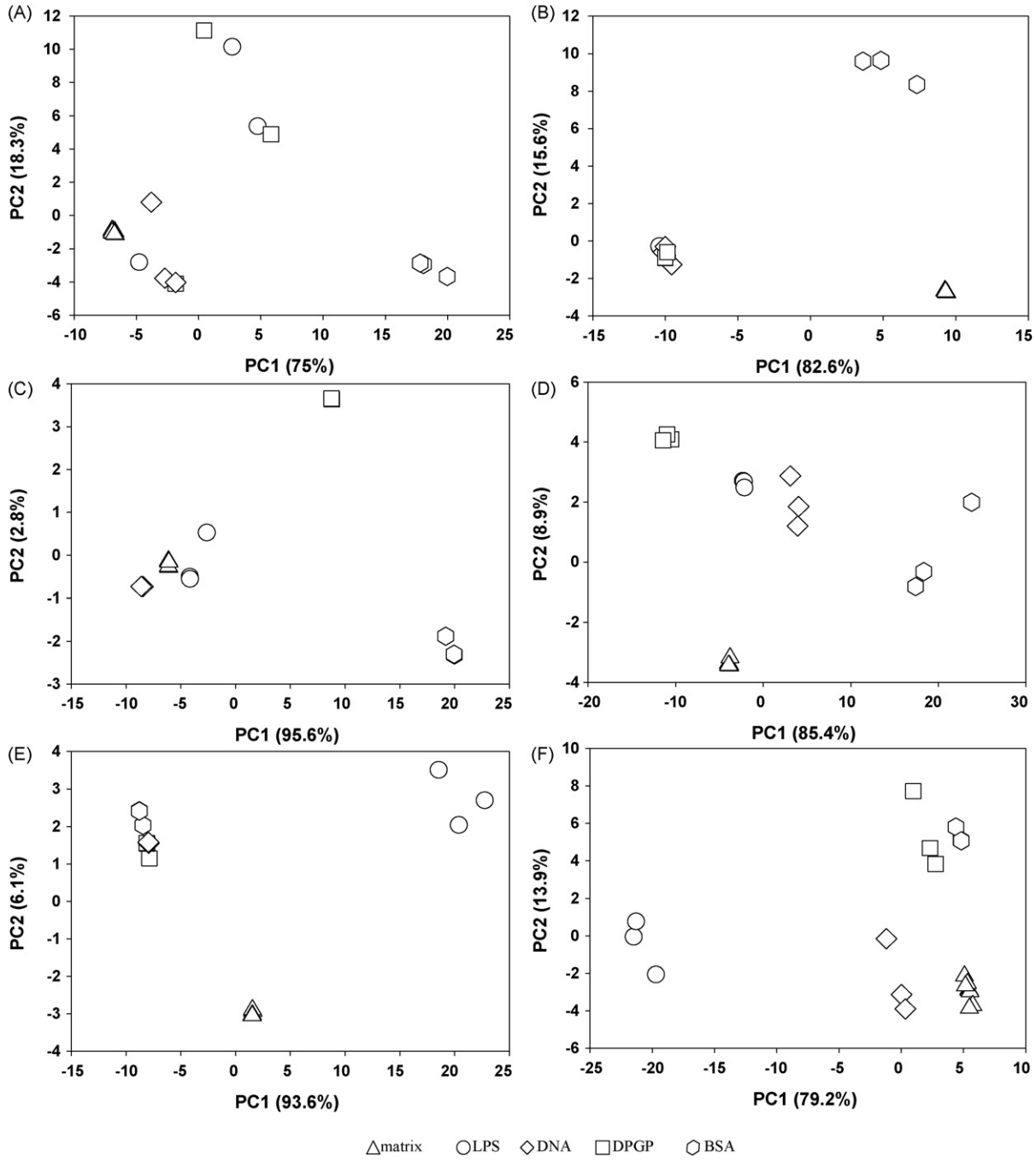

Fig. 2. 2D PCA plots corresponding to: Ni-dNTA (A and B), CAP18F (C and D) and ENP (E and F) sensors responses for: apyrogen water (A, C and E) and physiological saline solution (B, D and F). For analyte concentrations see Section 2.3.

variation due to the change of concentration of LPS is mainly manifested at low frequencies (Fig. S2), as observed by other authors for a sensor of similar characteristics (Giroud et al., 2009). For example at 1 Hz the change of the resistance values obtained for LPS concentrations of 0, 0.01, 0.1 and 1 ng mL^{-1}, are 407, 1407, 1533 and 1686 Ω, respectively. We consider that further improvements in the sensitivity and robustness of this setup need to be achieved as a quantitative tool covering a wide range of concentrations.

3.6. Combined two-sensors array and binary mixture analysis

Another approach investigated was working jointly the data provided by the second and third sensors on the same samples. A visual inspection of the PCA graph obtained for the combined analysis of the 204 data bits of information resulting from the impedance spectra obtained for the same sample using the CAP18F and ENP sensors, suggest a very good discrimination of all the different microbiological contaminants (Fig. 3 depicts the results for apyrogen water). Cluster Analysis was performed in order to quantitatively confirm the excellent grouping obtained by visual inspection in PCA. In fact, a 100% of correctly clusterized samples were obtained when CA was performed for a number of 82 samples, that is, all the samples with the same composition were assigned within a same cluster with no mistakes. CA provides also the so-called *silhouette* of each cluster, a function ranging from −1 to 1 (see Supplementary material); a value close to 1 means a good cluster-

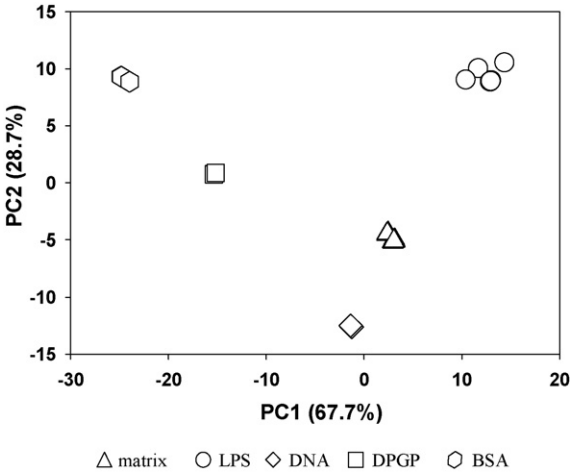

Fig. 3. 2D PCA plot corresponding to the combination of CAP18F and ENP sensor responses for apyrogen water. Samples are the same than in Fig. 2.

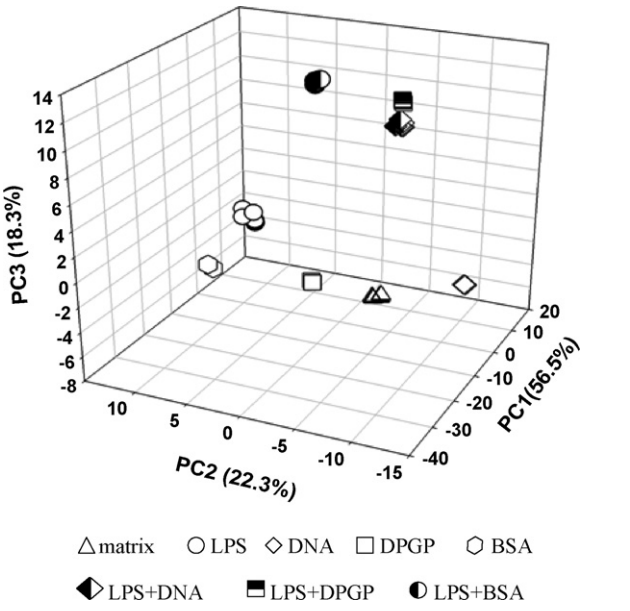

Fig. 4. 3D PCA plot corresponding to the combination of CAP18F and ENP sensors responses for apyrogen water contaminated with one analyte or a mixture of LPS plus other analyte. For analyte concentrations see Section 2.3.

ization. In practice, the optimal clusterization is given by similar *silhouette* values for the different samples within the same cluster, and by values close to 1 of its average, referred as the average silhouette width, ASW. Values of ASW between 0.7 and 1 are considered to indicate a good clusterization (Kaufman and Rousseeuw, 1987). In this case an ASW of 0.98 is obtained, confirming the goodness of the clusterization (ASW details are given in Table S1). Similar results were obtained for the physiological saline solution.

Further experiments were carried out, in which the CAP18F and the ENP sensors were applied to samples containing 0.1 ng mL^{-1} LPS mixed with increasing amounts of BSA, DNA and a phospholipid (DPGP); in some cases with concentrations 100 times higher than LPS. The samples tested were LPS, BSA, DNA, DPGP, LPS + BSA, LPS + DNA and LPS + DPGP.

As a first step, the impedance spectra of each sensor were individually analyzed by PCA. The results for the CAP18F sensor shows that the samples containing BSA present very important differences respect to the others (Fig. S3-top). However, the sample that contains a mixture of BSA and LPS can be easily distinguished at first sight, differentiating it from the sample contaminated only BSA. CA shows that the system is able to successfully discriminate six groups: pure apyrogen water, LPS, DPGP, BSA, LPS + BSA and a group containing LPS + DNA, LSP + DPGP and DNA with an ASW of 0.86 (ASW details are given in Table S2).

On the other hand, the PCA plot for the ENP sensor discriminates the samples in four well defined groups (Fig. S3-bottom), one corresponding to LPS, the other to pure apyrogen water, a third one containing the data corresponding to BSA, DNA and DPGP, and a forth one for LPS samples mixed with the other impurities. The 4 groups can be perfectly discriminated by cluster analysis given an ASW of 0.97 (ASW details are given in Table S2), representing a powerful tool for the detection of low LPS concentrations even in the presence of other contaminants in concentrations 100 times higher.

Thus, combining the impedance data from the two sensors, PCA allows the separation of the all type of samples (Fig. 4). CA is able to discriminate 7 groups: apyrogen water, LPS, DNA, BSA, DPGP, LPS + BSA, and LPS contaminated with substances containing phosphate moieties (DNA and DPGP) with an ASW of 0.96 (ASW details are given in Table S3).

We have mentioned that the method was primarily thought as an alarm test able to signal endotoxin concentration above threshold limits in presence of other microbiological contamination. A second target was to extend the identification capacity to the other possible contaminants present in the matrix. By introducing previous training, the capacity of the system also to classify samples was explored. The samples were classified using MDA. According with this procedure, supervised multivariate methods can be used to train the sensor array to recognize relevant contaminants. 83 samples were randomly chosen to train the system and 23 as testing samples. This process was randomly repeated three times and in all the cases 100% of correct classification of the testing samples was obtained.

An analysis of the main components allows choosing the most significant frequencies for a successful result. It can be observed that working with frequencies among 0.1 and 1 Hz, and from 500 to 1200 Hz a similar plot is obtained (Fig. S4). The number of necessary frequencies used is thus reduced to 14, which appears as a potentially very interesting simplification.

4. Conclusions

This work shows that endotoxin detection at very low concentrations, and discrimination from possible other contaminating species of microbiological origin can be carried out, by analyzing the impedance response using suitable modified electrodes. Three types of electrodes were studied. The first one, modified with a Ni complex (Ni-dNTA), is able to interact selectively with compounds containing phosphate groups, yet showing a limited capability for the objectives of this work. The second electrode, in which the Ni-dNTA layer is used to bind a peptide (CAP18F) able to recognize the lipid A moiety of LPS, shows some discriminating improvements, but the presence of LPS is sometimes not distinguishable from apyrogen water. The third electrode, modified with ENP, can discriminate LPS from the other contaminants; however, the rest of them cannot efficiently be differentiated from each other. A combined analysis of the data collected using both the second and the third electrode, discriminates efficiently all the contaminants and their binary mixtures.

The investigated method has several interesting potential advantages: it allows the detection of the tested contaminants without the need of sample conditioning, rinsing steps, or addition of probes; the incubation time is of only 15 min. The fact that the sensors can be constructed and modified in batches guaranties

the reproducible of the assay without the use of references. Due to the very short separation between electrodes, the sensor system can work in environments of very low conductivity (mainly, purified apyrogen water). The surface modification and conditioning by electropolymerization, and further selective reactions, can be easily adapted to a nanoscale system.

The combined sensor array has shown to be a very sensitive tool for LPS detection, and able to discriminate solutions containing 10 pg mL^{-1}, equivalent to 0.03 EU mL^{-1} measured by LAL test, providing a useful threshold alarm test for endotoxin detection, equalizing, or even surpassing, specified levels established by the most strict regulations (United States Pharmacopoeia 30, 2007; Nystrand, 2008).

Finally, it is worth to be noted that standard assays currently in use for checking out contamination from bacterial decomposition residues, focused essentially on LPS and assumes that this specimen alone is an integral indicator of presence (or absence) of pro-inflammatory impurities, overlooking a substantial percentage of other species of bacterial origin, such as nucleic acids, which recent publications point out as probable pyrogenic candidates (Schindler et al., 2004), so far underestimated. The presented method proves to be able to detect, in low concentrations and in mixtures, other biomolecules such as proteins, nucleic acids and phospholipids, opening a new route for controlling a broader range of potentially pro-inflammatory molecules of microbial origin at very low concentrations in ultrapure water; an issue that is of current concern for the hemodialysis community utilizing high-flux last generation dialysis membranes and On Line Hemodiafiltration (OLHDF) techniques.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.04.004.

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