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Toward Early Diagnosis of Late-Onset Sepsis in Preterm Neonates: Dual Magnetoimmunosensor for Simultaneous Procalcitonin and C-Reactive Protein Determination in Diagnosed Clinical Samples.

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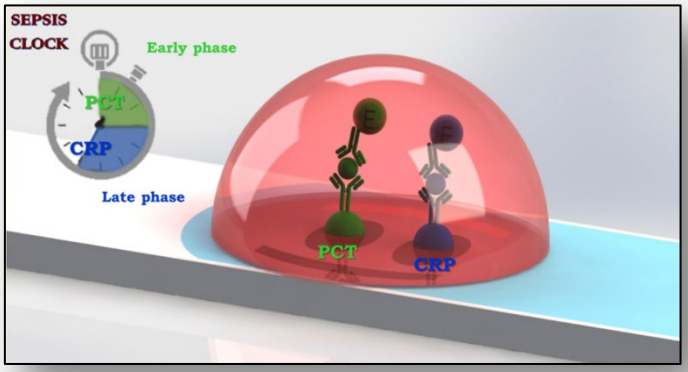
Abstract



Early diagnosis of sepsis, combining blood cultures and inflammation biomarkers, continues to be a challenge, especially in very low birth weight (VLBW) infants because of limited availability of blood samples. Traditional diagnostic procedures are cumbersome, not fast enough, and require relatively large volumes of sample. Empiric use of antibiotics, before diagnostic confirmation, is required to decrease mortality, leading to potential antibiotic resistance and side effects in VLBW infants. To solve such a serious problem, a dual magneto-immunosensor is proposed for simultaneous assessment of two of the most important sepsis biomarkers: procalcitonin (PCT for early phase) and C-reactive protein (CRP for late phase). This “sample-to-result” approach exhibited excellent sensitivity, selectivity, precision, and stability using low sample volumes (< 30 μL) and under 20 min of total assay. The analytical usefulness of the approach was demonstrated by analyzing clinically relevant samples of preterm neonates with suspicion of sepsis.

1. Introduction

Sepsis is defined as a syndrome of systemic inflammatory response syndrome (SIRS) following the passage of pathogenic microorganisms into the bloodstream. This systemic body infection is triggered by pathogens like bacteria, viruses, fungi, or parasites.[1,2] Predisposing factors for sepsis are young age (newborns), old age, AIDS, kidney or liver failure, antirejection therapy after organ transplantation, and special medical conditions.

Neonatal sepsis remains one of the leading causes of morbidity and mortality among both term and preterm infants, being especially problematic in very low birth weight (VLBW) (< 1500 g) neonates.[3] Treatment delay is associated with increased mortality. However, early recognition and diagnosis of neonatal sepsis is difficult because of the variable and nonspecific signs and symptoms.[4] Despite the low sensitivity and delayed availability, blood cultures still constitute the gold standard in the diagnosis of sepsis.[5] For that, among other clinical symptoms, sepsis suspicion and treatment initiation are based on the analysis of biomarkers as C-reactive protein (CRP), cytokines, and procalcitonin (PCT).[6,7]

PCT is an endogenous, nonsteroidal, and anti-inflammatory molecule containing a 116 amino acid peptide chain with a molecular weight of 13 kDa.[8] This molecule derives from the precursor preprocalcitonin (141 amino acid), and is the precursor of calcitonin, a hormone involved in calcium homeostasis which is also used as a thyroid cancer marker.[9]

However, during severe infectious disease and sepsis, PCT levels can be rapidly increased in other tissues such as the lung and intestinal cells in response to pro-inflammatory stimulation. Under normal physiological conditions, PCT concentration in blood is lower than 0.25 ng/mL, but elevated concentrations could be used as a reliable positive evaluation of the severity of sepsis. Values of PCT between 0.25 and 0.5 ng/mL are related to mild or local bacterial infection; values from 0.5 to 1 ng/mL suggest infection or septicemia; and from 1 to 100 ng/mL indicate systemic bacterial infection.[10] PCT levels increase rapidly after 2−6 h of sepsis development and reach the maximum level after 8−24 h, with a half-life of 24 h.[11] The PCT levels will decrease within the normal range if the

infection disappears. Therefore, the measurement of PCT levels could be used for early diagnosis as well as for the evaluation of the treatment effectiveness.[12−14]

On the other hand, CRP (α-globulin of 120 kDa) is an acute phase reactant, produced by the liver during inflammation, produced by invasion of microorganisms, or in the presence of tissue damage.[15,16] Its levels begin to rise 10−12 h after infection, and this make it a late biomarker of neonatal infections. Values higher than 10 μg/mL are considered positive for sepsis diagnosis. CRP has also been shown to be a good marker of the response to antibiotic therapy.[17−19] **Figure III.2.1** shows PCT and CRP kinetics schematically after pathogen exposure and their utility in sepsis diagnosis and monitoring.

Standard assays to detect sepsis biomarkers, such as chemiluminescence immunoassay (CLIA), fluorescence immunoassay (FIA), enzyme-linked immunosorbent assay (ELISA), and immunoturbidimetric assay, are sensitive but usually suffer from important sample and reagent consumption. Moreover, routinely, they are performed in central laboratories and take a long time, which is highly incompatible with the need of having quick decisions. A reliable, quick, sensitive, and specific test for sepsis diagnosis at the bedside is needed in order to obtain adequate information for the correct indication of antimicrobial therapy. This may improve patient outcomes and avoid invasive procedures and unnecessary exposure to antibiotics, preventing the promotion of drug resistance of bacteria, long hospitalization, and increasing health costs.[7,12]

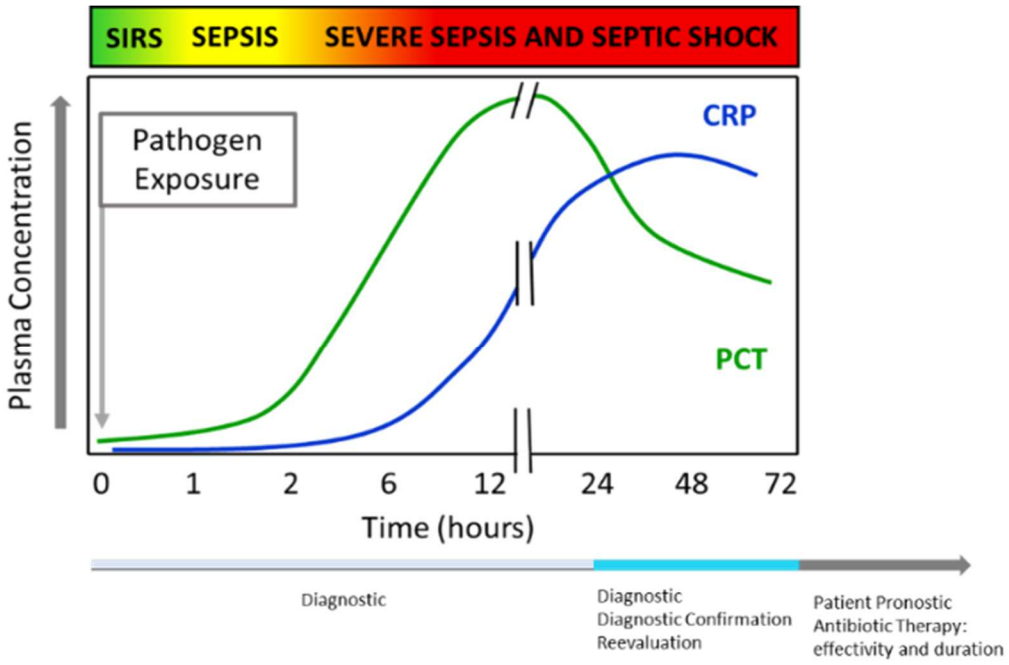


Figure III.2.1. PCT and CRP kinetics after pathogen exposure and utility in sepsis diagnostics and monitoring.

Due to the limited specificity, the measurement of a single sepsis biomarker is usually not enough to diagnose sepsis. In this sense, multiplexed assays, which allow the simultaneous detection of several sepsis biomarkers, are attracting much attention to provide a more accurate diagnostic tool and significantly enhance the predictive value for the risk of sepsis.[20–22] In this regard, the usefulness of the PCT/CRP ratio in various infectious conditions of adult patients has been studied.[23] Multiplexed immunosensors are advantageous because they offer higher sample throughput, simplified analytical procedures, less sample consumption, reduced turnaround times, improved test efficiency, and more reasonable cost compared to traditional in-parallel single analyte immunoassays.[7,24,25]

Electrochemical immunosensors can be envisaged as a valid alternative to classical methods of analysis for clinical biomarkers offering the advantages of being easy to use, rapid, robust, often inexpensive, and capable of multianalyte testing and useful to facilitate *point-of-care-testing* (POCT).[26] Although some electrochemical immunosensors have been proposed for the individual determination of CRP[27–32] and PCT,[33] to the best of our knowledge, there are no publications of simultaneous determination of CRP and PCT or for the analysis

of neonatal clinical samples with high significance and limited availability. In addition, they do not explore the analysis of these sepsis targets in clinical samples.

In this work, we describe the first dual electrochemical magnetoimmunosensor for fast and simultaneous detection of target protein sepsis biomarkers (PCT and CRP) in preterm neonates using a small volume of samples.

2. Experimental section

2.1. Reagent and solutions

CRP (8C72), together with the corresponding paired monoclonal mouse anti-human CRP antibodies (4C28C HRP-conjugated and 4C28B biotinylated), lyophilized human PCT (8PC5) and two paired monoclonal mouse antihuman PCT antibodies (44D9 HRP-conjugated and 18B7 biotinylated) were obtained from HyTest (Turku, Finland).

The lyophilized PCT was reconstituted in deionized water (Millipore Milli-Q purification system). Dilution of CRP, PCT, and antibodies were prepared in PBST buffer solution pH 7.5 (0.1 M phosphate, 0.138 M NaCl, 2.7 mM KCl (Scharlau), and 0.01% Tween 20 (Sigma-Aldrich)).

Ethylenediaminetetraacetic acid (EDTA), sodium citrate, and heparin solution were obtained from Sigma-Aldrich (Madrid, Spain). Their solutions were prepared with PBST buffer solution pH 7.5.

Enzyme substrate PERDROGEN (30% H_2O_2), hydroquinone, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Madrid, Spain). Magnetic beads functionalized with streptavidin (Dynabeads® M-280 Streptavidin) were purchased from Invitrogen (Carlsbad, CA). Hydroquinone and peroxide solutions were prepared in phosphate buffer 0.1 M, pH 7 (PB).

2.2. Samples

Plasma sample from a healthy donor was obtained. Hospital Clínico San Carlos (Madrid, Spain) provided unique plasma samples from neonates coming from healthy and sick individuals, including extremely preterm and low birth

weight infants. The study was approved by the Ethics Committee of the Hospital and parental informed consent was obtained before the collections of blood samples. Babies were included in the study only if blood samples were needed as part of the standard care in the intensive care unit and not just for the purpose of the present study. Samples were obtained by venipuncture. 500 µL of whole blood was collected in a heparinized tube and transported to the central laboratory of Hospital Clínico San Carlos for standard analysis (BRAHMS CRPus KRYPTOR and BRAHMS PCTsensitive KRYPTOR). Another aliquot of 500 µL of whole blood was collected in a heparinized tube and centrifuged at 1000 rpm for 15 min. The supernatant was separated and transported to the research laboratory on ice. Due to the special characteristics of these patients (low or extremely low birth weight), the available sample volume is very low, and only tens of microliters can be used for each different replication.

For CRP determination with our approach, a previous sample dilution 1/100 in PBST was performed, due to the high concentration levels presents in the samples. In the case of PCT determination, sample dilution was not carried out.

2.3. Apparatus and electrodes

Amperometric measurements were carried out using a multi potentiostat/galvanostat µSTAT 8000 from DropSens, Oviedo (Spain), which incorporates the software “Drop-View 8400”. Dual screen-printed carbon electrodes (dSPCEs) (DRP-1110, Dropsens) consisting of two elliptic carbon working electrodes (6.3 mm^2 each) including a silver pseudoreference electrode and a carbon counter electrode were used.

Advanced Vortex Mixer-ZX3 from Velp Scientifica and Pulsing Vortex Mixer from VWR Signature were used for incubation stages. Magnetic block DynaMag™-2 was obtained from ThermoFisher and used for handling of magnetic particles, and a homemade magnet holding block to control the attraction of magnetic beads to the dSPCE surface was also used.

2.4. Immunoassay procedure

Inmunoanalytical methodology was developed based on an ELISA sandwich scheme, where biotinylated capture antibody was immobilized on streptavidin-functionalized MBs and detection antibody was labeled with HRP enzyme. It is

well known that using MBs as immobilization support entails remarkable benefits such as higher surface area, faster kinetic assay, diminishing of matrix effects, and facility of manipulation. By using these skills, both assays were performed in two microcentrifuge tubes, where the affinity reaction takes place. Furthermore, their combination with a dual working electrode on the surface of an SPCE, as disposable and portable electrochemical platforms, allows the simultaneous electrochemical transduction. This strategy improves sensitivity and electron transfer while diminishing the nonspecific adsorption, since affinity reaction is not performed on the electrode surface.

In this sense, 2 µL of a commercial streptavidin-functionalized MBs suspension of 10 mg/mL was transferred to both microcentrifugue tubes, washed according to the manufacturer's protocol and resuspended in 50 µL of a 1.4 µg/mL biotinylated anti-human CRP antibody and 5.0 µg/mL biotinylated anti-human PCT antibody solutions, prepared with PBST buffer, pH 7.5. After incubation at 25 °C under stirring during 15 min for CRP antibody and 5 min for PCT antibody, the tubes were placed on the magnetic holding block and the supernatant was removed. Immediately, two washing steps with 100 µL of PBST buffer were made. For anti-CRP modified MBs, a sequential incubation with the analyte and the labeled antibody was performed. Consequently, two incubation stages of 5 min at 25 °C with 50 µL of defined CRP concentration or sample and 50 µL of HRP-labeled antibody (0.6 µg/mL), respectively, were carried out. Finally, the supernatant was removed followed by three washing steps. In the case of anti-PCT modified MBs, a simultaneous incubation with PCT standard or sample plus HRP-labeled antibody (0.4 µg/mL), 25 µL each at 25 °C for 15 min, was performed. After removing supernatant, three washing steps were carried out.

2.5. Electrochemical measurements

Once the immunoaffinity interactions were complete, both biomarker concentration related signals can be determined simultaneously on the surface of a dual electrode. The MBs complexes were resuspended in 5 µL of hydroquinone solution (1 mM) and magnetically captured on the working electrodes of the dSPCE (WE_1 is always used for CRP, and WE_2 is used for PCT).

Subsequently, 35 µL of hydroquinone solution (1 mM) was dropped covering the two working electrodes as well as the counter and pseudoreference electrodes.

Amperometric measurements were performed at −0.20 V after addition of 5 µL of hydrogen peroxide solution (5 mM final concentration). Signals calculated as the difference between the steady state and the background currents after 60 s were fitted to the fourparameter logistic equation (**Equation (III.2.1)**) using the software SigmaPlot 10.0.

$$\mathrm{ip} = \left(\frac{i_{max} - i_{min}}{1 + \left(\frac{EC_{50}}{x}\right)^{h}} + i_{min} \right) \quad \text{(III.2.1)}$$

where i_{max} and i_{min} are the maximum and minimum current values of the calibration graph, EC_{50} is the concentration of antigen that induces 50% of the maximum detectable signal, and h is the hill slope. The limit of detection (LOD) was calculated using the 3 sd/m criteria.

3. Results and discussion

Early sepsis diagnosis in preterm neonates is still a challenge and complex phenomenon involving the simultaneous determination of protein biomarkers such as PCT and CRP, among others, to improve diagnostic accuracy.

Traditional diagnostic procedures are cumbersome, not fast enough, and thus, urgent initiation of antibiotic therapy before confirmation of sepsis diagnosis is required to decrease mortality, leading to potential antibiotic resistance and severe side effects in newborns. The benefits of early diagnosis of sepsis include improved patient outcomes and decreased medical costs. In addition, there is a clear unmet medical need: the use of small sample volumes for multiplexed analysis and the serial analysis of the biomarkers that improve the diagnosis and the disease monitoring. To this end, a dual magnetoimmunosensor is proposed. The assay approach for simultaneous determination of two sepsis biomarkers is depicted in **Figure III.2.2**. The dual-simultaneous detection can be carried out using less than 30 µL of clinical sample from preterm neonates and short time

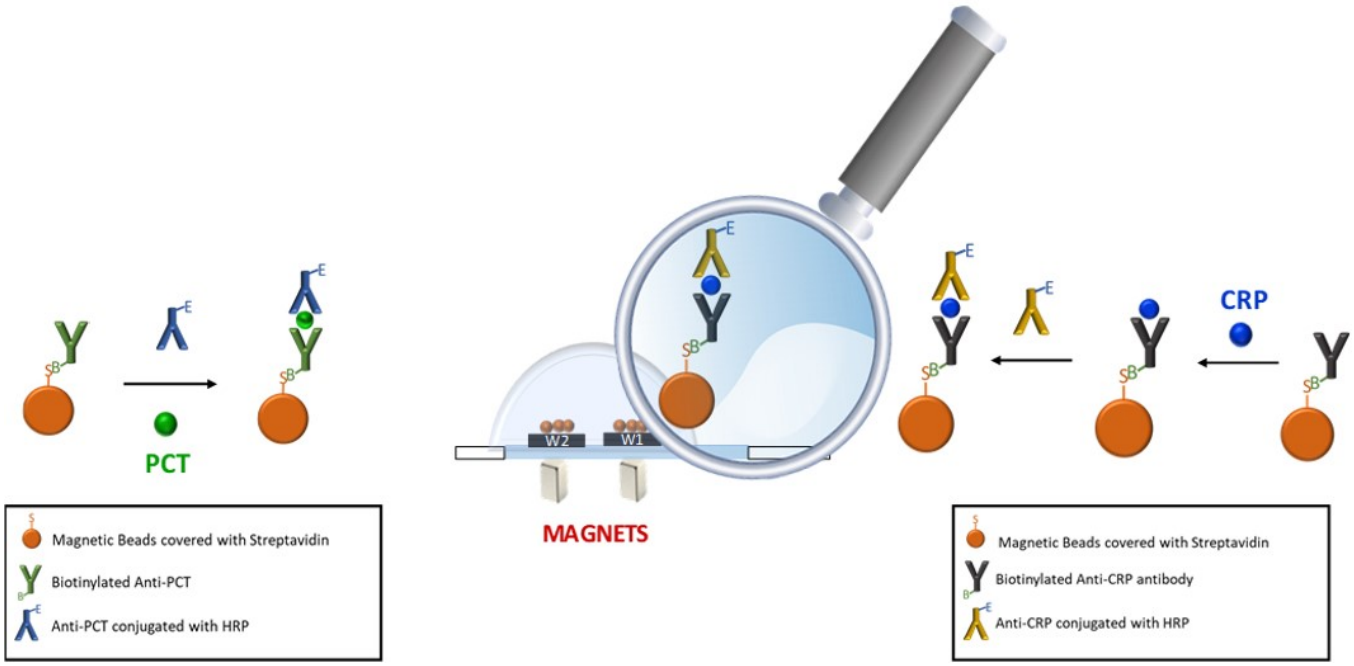


Figure III.2.2. Detailed schematic representation of the electrochemical magnetoimmunoassay strategy for the simultaneous detection of PCT and CRP.

(< 20 min). This is a critical aspect in neonatal patients with an extremely low blood volume and allows correct disease and treatment monitoring.

3.1 Analytical features for simultaneous determination of PCT and CRP

First, the influence of the amount of biotinylated capture antibodies and detection antibodies for both biomarkers were carefully studied. Capture antibodies immobilized onto the MBs were evaluated between 0 and 10.0 µg/mL and 0 and 5.4 µg/mL for PCT and CRP immunosensors, respectively. Detection antibodies labeled with HRP were tested in the range of 0−1.0 µg/mL for both assays. The selected values, which correspond to the saturation binding sites onto MBs and the best compromise in sensitivity and working range of PCT and CRP dual immunosensor, are summarized in **Table III.2.1.** In order to diminish the total assay time, the incubation mode (sequential or simultaneous) and incubation times were also studied. Simultaneous incubation during 15 min of the analyte and the detection antibody was selected for the PCT assay, while sequential incubation stages of 5 min were chosen for CRP assay. In both cases, nonspecific adsorption was below 2%. It is important to remark that the affinity assay is performed in the test tube, while detection takes places on the surface

of each working electrode of the dual sensor. Therefore, only addition of BSA 0.01% to the dilution buffer was carried out. Detection conditions were selected according to previous optimizations.[34] It is worth noting the low incubation times for both biomarkers, allowing simultaneous and fast determination.

Table III.2.1. Optimization of the experimental variable tested for simultaneous affinity reactions of PCT and CRP assays.

Parameter	PCT assay		CRP assay	
	Tested Range	Selected Value	Tested Range	Selected Value
[Capture antibody], µg/mL	0 – 10	5	0 – 5.4	1.4
Capture antibody incubation time, min	0 – 60	5	0 – 60	15
Protein incubation time, min	-----	-----	0 – 60	5
[Detection antibody], µg/mL	0 – 1	0.36	0 – 1	0.6
Detection antibody incubation time, min	----	----	0 – 60	5
Protein + Detection Ab incubation time, min	0 – 60	15	----	----

Figure III.2.3 shows the typical sigmoidal calibration curves of ELISA sandwich assays, obtained in each working electrode for simultaneous determination of CRP and PCT. **Table III.2.2** lists the working ranges and the LODs obtained (0.09 ng/mL PCT and 0.008 µg/mL CRP), which showed the suitability of this approach for the simultaneous determination of both biomarkers at clinically relevant concentrations.

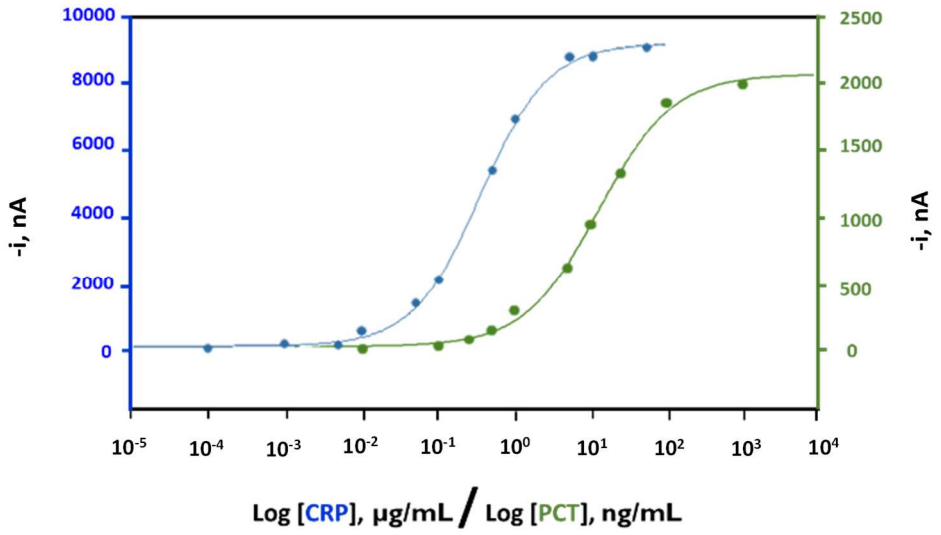


Figure III.2.3. Calibration curves for sepsis biomarkers: PCT and CRP.

The precision of the developed dual immunosensor was evaluated by assaying a concentration level for each biomarker (5 µg/mL for CRP and 100 ng/mL for PCT) in the same day (n = 8) and in different days (n = 5). The low variation coefficients (CV) obtained demonstrated the good repeatability and reproducibility of this dual immunosensor (see also **Table III.2.2**).

Table III.2.2. Analytical characteristics obtained for PCT and CRP assay using the dual magnetoimmunosensor.

Analytical Characteristic	**PCT assay**	**CRP assay**
r	0.999	0.997
EC_{50}	12 ± 1 ng/mL	0.34 ± 0.02 µg/mL
Working range	(0.25 - 100.0) ng/mL	(0.01 - 5.0) µg/mL
LOD	0.09 ng/mL	0.008 µg/mL
Precision Study	**PCT assay**	**CRP assay**
Intra-assay, CV%	7%	8%
Inter-assay, CV%	8%	8%
Stability Study	**PCT assay**	**CRP assay**
MBs + Capture Antibody	> 30 days	> 30 days

In addition, the promptness and ease of performing the analysis is a crucial aspect in the design of these assays. In this sense, the possibility to arrange some reagents in advance for in situ analysis has to be checked. Then, MB−captured antibody complexes were prepared, and their stability was monitored during a period of 30 days. All the MB−captured antibody complexes for each biomarker were stored in PBST buffer solution at 4 °C in independent microcentrifuge tubes. Successive measurements of 100 ng/mL of PCT and 5.0 µg/mL of CRP along this period gave a dual immunosensor response comprising the average and ±3 times the standard deviation of these signals, demonstrating the possibility of using these complexes as a kit reagent during more of 30 days (see also **Table III.2.2**).

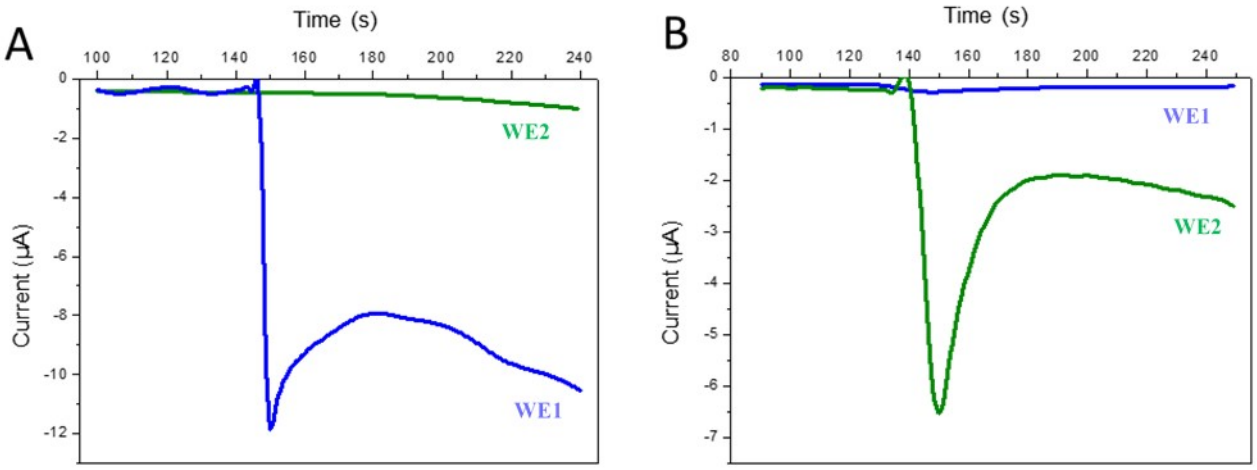


Figure III.2.4. Diffusional cross-talk between electrode assays. CRP (WE_1) and PCT (WE_2) simultaneous measurements in the d-SPCE. (A) Measurements of the signals produced by 5 µg/mL CRP and 0 ng/ mL PCT. (B) Measurements of the signals produced by 0 µg/mL CRP and 100 ng/mL PCT.

Considering that HRP enzyme is the common tracer for both immunosensors, appropriate control experiments were also carried out. Indeed, the two working electrodes are measuring the same enzymatic reaction product, which could lead to cross-talk between both electrodes. **Figure III.2.4A,B** shows the control signals produced in both working electrodes when one of the biomarkers is highly concentrated and the other is not present. This experiment demonstrates that at fixed measured time (60 s), the cross-talk by diffusion of the enzymatic reaction product between both working electrodes was negligible.

Also, selectivity from both immunosensors to its corresponding biomarker was checked in the presence of a large excess of the other biomarker (PCT or CRP) and other reagents commonly found in blood analysis such as heparin, EDTA, and citrate. As can be observed, there were no significant differences between the currents obtained in the absence and in the presence of these compounds, which demonstrated the excellent selectivity of the immunosensor for dual detection (**Figure III.2.5**).

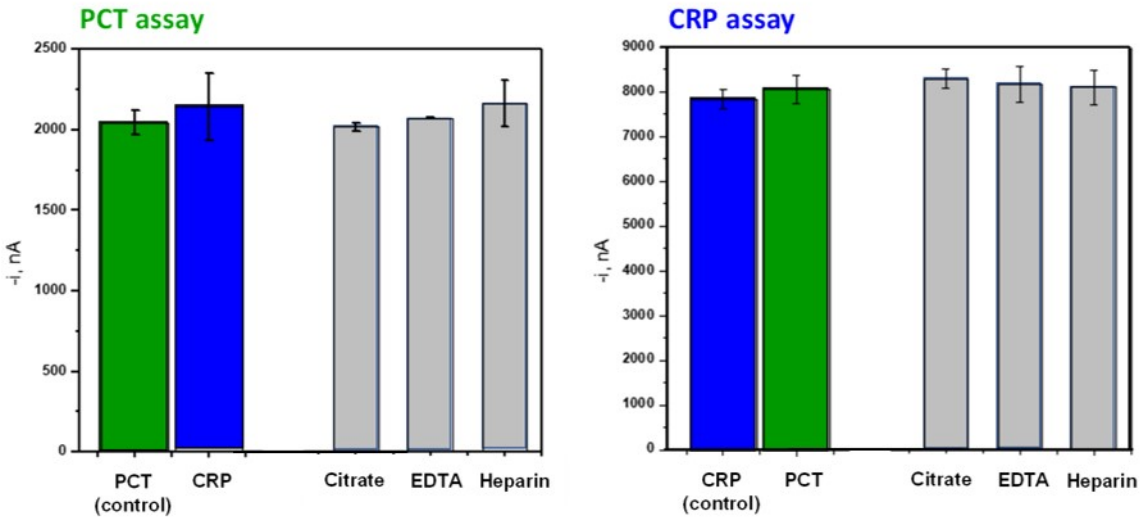


Figure III.2.5. Interference studies. PCT study on the left: signals obtained for PCT 10.0 ng/mL in buffer and in the presence of CRP (16 µg/mL), heparin (4 mg/mL), EDTA (1 mg/mL) and citrate (0.15 M). CRP study on the right: signals obtained for CRP 5.0 µg/mL in buffer and in the presence of PCT (1.0 µg/mL), heparin (4 mg/mL), EDTA (1 mg/mL), and citrate (0.15 M).

3.2 Analysis of plasma samples from preterm neonates

Before carrying out the analysis of plasma samples from neonates, the possible existence of a matrix effect was studied.

In that sense, for PCT determination, a calibration curve was directly constructed in the plasma of a healthy donor (concentration below the working range) spiking with different PCT concentrations. A statistical comparison between the slope values obtained using the plasma sample (1400 ± 100 nA ng^{-1} mL) with those obtained in PBS buffer (1370 ± 70 nA ng^{-1} mL) revealed no matrix effect ($p < 0.05$). It allowed working in the clinically relevant range without dilution.

However, CRP determination requires dilution of the sample since the patients would have a much higher concentration than the obtained working range (i.e., in the presence of infection, CRP levels can increase above 100 µg/mL).[35,36] Therefore, and because of the high sensitivity of the dual immunosensor, 1:100 dilution of plasma sample was used for CRP determination, avoiding the matrix effect and allowing the analysis of samples with concentrations throughout the clinically relevant range, from healthy to severe sepsis conditions.

Once the suitability of the proposed dual immunosensor has been demonstrated for determination of both biomarkers, plasma samples from

preterm neonates, including an extremely low (< 1000 g) birth weight infant, were analyzed in order to determine sepsis diagnosis. The samples were obtained from 3 neonatal ICU patients (Hospital Clínico San Carlos).

In **Table III.2.3**, the clinical characteristics of the patients and the sample analysis results obtained by the developed dual immunosensor as well as by the Hospital standard method are depicted.

Table III.2.3. Analysis of plasma samples from neonates with sepsis suspect using the dual magnetoimmunosensor developed and the Hospital standard method.

Patient	Diagnosis	Sample	Biomarker	Dual sensor determination	Hospital determination
Patient 1 Sex: male Gestational age: 25+4 weeks Birthweight: 900g	Extreme prematurity.	1	**CRP** µg/mL	73 ± 9	63.6
			PCT ng/mL	5.7 ± 0.8	4.5
		2	**CRP** µg/mL	39 ± 4	34.0
			PCT ng/mL	2.9 ± 0.2	3.1
Patient 2 Sex: female Gestational age: 35+3 weeks Birthweight: 1660g (First twin)	Intestinal perforation. Unproven early onset sepsis. Intrauterine growth restriction.	3	**CRP** µg/mL	37 ± 6	51.3
			PCT ng/mL	18 ± 1	21.0
Patient 3 Sex: female Gestational age: 35+3 weeks Birthweight: 2290g (Second twin)	Prematurity. Healthy control	4	**CRP** µg/mL	1.7 ± 0.1	< 2.9
			PCT ng/mL	0.6 ± 0.1	0.4

It is worth describing the peculiarities of the patients' conditions in order to understand the significance of the analyzed samples. Samples 1 and 2 proceed from patient 1, who due to his extreme prematurity and the appearance of some sepsis symptoms was analyzed before and after antibiotic therapy. In this sense, not only the diagnosis but also the evolution assessment was properly performed. Samples 3 and 4 proceed from twins, one of them suspected of suffering from sepsis (patient 3) and a healthy control (patient 4).

Interestingly, in light of the enormous difficulty in the simultaneous reliable assessment of both sepsis biomarkers, due to the high difference in the

concentrations during sepsis evolution, sample scarcity, as well the inherent complexity of the sepsis, these results demonstrated the suitability of the dual immunosensor. Comparison of the results obtained by our dual immunosensor and those obtained using the reference methodology at the Hospital (BRAHMS CRPus KRYPTOR and BRAHMS PCT sensitive KRYPTOR) did not show statistically significant differences ($p < 0.05$). Only a minimum divergence in the CRP sample from patient 2 was observed, revealing a high agreement between the results obtained in this work and those declared by the Hospital. It is worth noting that sepsis diagnosis was afforded with success in a critical group of patients using precious and unique real samples.

4. Conclusions

In this work, a dual (and disposable) electrochemical magnetoimmunosensor, on a screen-printed platform, for the simultaneous determination of PCT and CRP plasma samples from preterm neonates, has been reported for the first time. It is important to highlight that simultaneous determination of both CRP and PCT biomarkers is not easy because they are time-dependent diagnostic biomarkers with a high difference in levels of concentration.

The dual immunosensor exhibited an excellent analytical performance in terms of sensitivity, selectivity, precision, and stability, using low sample volumes. The achieved performance makes the developed dual sensor a useful and affordable analytical tool for the rapid and simultaneous determination of PCT and CRP biomarkers as relevant indicators for sepsis diagnosis and therapy monitoring. Its evaluation through the measurement of precious real samples from neonates demonstrates its applicability as a low-cost, easy-to-use, and reliable alternative diagnostic tool for onsite/bedside clinical analysis. These results make our device a highly competitive approach in this field and draw new hope in the complex landscape of sepsis diagnosis for preterm neonates and all patients in general.

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Authors contribution

Á.M.-F. and M.M.-G. contributed equally. The manuscript was written through contributions of all the authors. All the authors have given approval to the final version of the manuscript.

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