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Monitoring the level of infection by COVID-19: an previous experiment to possibility of future application to the C-reactive protein detection by bioelectric signals

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Abstract: C-reactive protein (CRP) is a marker of inflammation and infection, and is altered in COVID-19 patients. 2-methacryloyloxyetyl phosphorylcholine (MPC) is a polymer containing phosphorylcholine, a protein that anchors CRP. The purpose of this work was to detect CRP by bioelectric signal resulting from its interaction with MPC. The signal acquisition system was elaborated using Arduino in conjunction with the Parallax Data Acquisition (PLX-DAQ) program for data transfer to Excel, which allowed the treatment of the obtained signal. 10 volunteers were also enrolled to provide blood samples for the purpose of using CRP on confectioned biomaterial containing MPC. After pipetting the volunteer's blood serum into the biomaterial containing MPC, it was possible to obtain a bioelectric signals from the interaction of MPC with CRP. It is concluded that it is possible to detect the presence of CRP by bioelectric signal, and that the use of MPC is promising for future application in collection strips, which would allow the quantification of CRP by portable electronic equipment. An application example would be monitoring the infection level of patients with COVID-19.

Keywords. *Biomedical engineering, biomaterials, inflammation, infection, SARS-CoV-2.*

Introduction. CRP is an acute-phase protein of inflammation, but it is also present in cases of chronic inflammation. The classic action of CRP is made by its binding to a membrane anchor protein called phosphorylcholine (phosphocholine), and then this phosphorylcholine-CRP complex induces C1q (complement system protein) fixation, thus stimulating opsonization and phagocytosis of offending agents through the interaction of CRP with macrophages/monocytes, with consequent secretion of pro-inflammatory cytokines. It turns out that CRP also decreases neutrophil actions, due to its degradation by lysosomal enzymes, thus CRP is considered an inflammation regulating protein, for its pro and anti-inflammatory actions (1).

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It is noteworthy that the methods for CRP measurement evolved over time, and the pioneer method was qualitative by capillary tube precipitation reaction, later emerged the semi-quantitative method by latex agglutination, both methods of limited clinical value, and more recently, quantitative immunological methods have emerged, such as turbidimetry (immunoturbidimetry) (1).

The measurement of CRP is usually used in several clinical situations, especially in the following clinical conditions and pathologies: pneumonia, acute otitis media, urinary tract infection, osteomyelitis, meningitis, sepsis, appendicitis, pelvic inflammatory disease, acute pancreatitis, postoperative and burnt patients follow-up, cancer, rheumatologic diseases, Crohn's disease, ulcerative colitis, atherosclerotic disease (1).

Patients with COVID-19 have changes in CRP levels, including ranges of specific values according to the level of disease, including correlation with reduced oxygen saturation (2-10).

The creation of MPC was considered a milestone in the development of biomaterials by solving the problem of physical and chemical instability of phospholipid membranes used as drug carriers, sensors and separation membranes. MPC monomers are hydrophilic, but when they are copolymerized with other monomers, these hydrophobic form hydrogels (11).

MPC is now being used in research of various segments in the line of biomaterials, bioengineering and biomedical engineering, due to its characteristics that result in biocompatibility, as per applications listed below.

In the oral health field, MPC has proved to be effective in suppressing oral bacteria in mouthwash application in healthy subjects (12), it has proved to have antibacterial effect when incorporated into calcium silicate cement used in endodontic procedures (13), reduction in protein absorption to one tenth and reduction to one-fifth of periodontitis-related pathogen metabolic activity caused by dental root caries treatment (14), showed a preventive effect for dental enamel demineralization (15).

As for orthopedics, MPC was effective in reducing cellular connection in hip titanium implants without impairing mechanical performance (16), as well as being used as a specific drug delivery

agent for lubrication of joint cartilage affected by titanium nanotube, with possible future application in titanium implants (17).

MPC also demonstrated hematological compatibility by not inducing increased blood coagulation in in vitro and in vivo models (18), as well as being efficient in promoting endothelial proliferation and reducing platelet adhesion in a pharmacological catheter introduced into the human umbilical cord vein (19), even when MPC was introduced on the surfaces of ventricular assist devices, it showed biocompatibility, with reduced thrombogenic deposition and reduced platelet activation (20, 21).

Another interesting application of MPC is as a carrier of doxorubicin, a drug administered for cancer treatment. At the time rats that had cancer cell cultures implemented were used, and MPC was conjugated with other substances to yield three doxorubicin-loading composites. The results showed a reduction in tumor size, with a marked reduction in toxicity, since the action of the drug occurred locally, with low concentrations in the animals' blood (22).

When MPC is polymerized, it is common for its nomenclature to become PMPC.

In addition to these applications, few studies have attempted to detect CRP by its binding to MPC in bioelectric signaling devices, which leaves a gap in the literature. However, further progress in this field of technological development may contribute to the emergence of reagent strip user equipment for real-time CRP dosing, which could be beneficial to the market and society as there would be many applications.

Therefore, the objective of this study was to detect the presence of CRP by bioelectric signal.

Matherials and methods. This work has been divided into three parts: 1) development of the material, with applied research characteristic; 2) development of bioelectric signal acquisition system, with applied research characteristic; 3) obtaining bioelectric signal in the presence of CRP, with volunteer blood deposited in a collection strip containing the reagent material (MPC + silica), with experimental research characteristic.

For the collection, venipuncture was adopted, with vacuum collector, obtaining approximately 5 ml of blood in each collection. After 20 minutes of blood collection, centrifugation was

performed at 3000 rpm for 5 minutes (Model 80-2B, Centribio, São Paulo, Brazil), obtaining the separation of solid content of blood serum.

In order to keep the blood sample in a good state of conservation, it was kept cool after collection, after centrifugation and during transport for storage in a refrigerator, until it was transported to the IFSP - Itapetininga chemistry laboratory to be used for testing of the proposed material and system by pipetting in the strips containing the reagent material.

The reagent material was developed in the IFSP - Itapetininga campus chemistry laboratory, from MPC and silica, deposited on the collection strip, and it was possible to measure the bioelectric signal generated by the reaction between the blood CRP and the phosphorylcholine contained in MPC.

Initially, the MPC polymerization process and SiO2 addition were carried out, following the process below:

1- Weighing of MPC monomers using BEL Engineering® M214Ai precision scale, weighing 558 mg of MPC (3 x 0.186 grams);

2- MPC transfer to three self-cleavable vials;

3- Weighing of silica, taking into account the 2:1 molar ratio (silica:MPC), with silica having a molar mass of 60.08 g/mol and MPC of 295.27 g/mol, obtaining 75.6 mg of silica in each vial (total = 226.8 mg);

4- Addition of silica in the ratio 2 silica to 1 MPC;

5- Addition of 60 milliliters of ethanol (99.5% content) in each vial;

6- Vial deposit in the oven (Solab® - model SL-100) at 60 degrees celsius to remain for six hours;

7- Natural cooling.

After this process, the material was recrystallized, so that it was possible to deposit the final product (reagent material) in the blank strips, and for this the following steps were performed:

1- Preparation of a mixture of ether and chloroform in the ratio of 7/3;

2- Transfer of this mixture to the polymer synthesis solution (600 ml ether/chloroform to 60 ml reagent material);

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3- Removal of supernatant;

4- Transfer to tubes

5- Centrifugation for solid material separation, using KASVI® K14-4000 centrifuge, for 20 minutes at 4000 rpm.

At the same time as the process described above was undergoing, the blank strip was made, still without the deposit of the reagent material in its final product form. For this purpose, a model was printed on sulfite paper, and later aluminum foil was added to function as the electric conducting electrodes, in the place of the strip with dimensions of 32 mm x 5 mm, leaving a space of 2 mm x 5 mm in the center for addition of the reagent material.

After this step, 10 microliters of the reagent material (MPC + SiO2) were added through pipetting into the central space described above and oven drying was performed for 10 minutes at 50 degrees celsius.

Acquisition of the bioelectric signals occurred through a system containing an Arduino© (Arduno Uno - microcontroller) board, program development environment (Arduino IDE®), and Parallax Data Acquisition (PLX-DAQ)® software from Parallax Inc© to transfer data obtained by Arduino to Excel spreadsheet, and thus perform mathematical modeling of the signal obtained.

Thus, this system converted the input analog bioelectric signal into a digital signal (discrete - uniform rate sampling) and, subsequently, this converted signal (analog-digital) was processed digitally (finite number of discrete samples), and the analog input signal manipulation was performed by an electronic circuit, resulting in a biomedical signal processing system (23), the scope of this work.

From this, the area of each bioelectric signal can be calculated by calculating the integral using the approximation by the sum of the heights of the rectangles on the left, as recommended by Rogawski (24), using the formula $LN = \Delta x \sum_{j=0}^{N-1} [\![f(a+j\Delta x)]\!]$, with read rate of 1 millisecond (1ms).

This study was approved by IFSP - São Paulo Ethics Committee, having its performance approved according to opinion number 3.417.909.

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10 healthy athletes volunteers was recruited pre, immediately post and 48 hours post one training session, as a way to generate immune stress and obtain different CRP quantities and different bioelectric signals.

This model was a resource to simulate an little infection, to make this experiment an safely process.

All they signed an informed consent form (ICF) to ensure that only data related to the experiments was used for the research in question and for future publications, and thus personal data was protected by confidentiality.

For blood collection, new, packaged, and disposable materials were used. Disposal of the perforating and contaminating material at the time of collection was made through the use of its own box (decarpack). It is noteworthy that the entire procedure was performed by a qualified professional and making use of biosafety standards.

For the test, 20 microliters of serum content of the blood sample were pipetted into the reagent strip.

The amount of 20 microliters of the serum content has been established as consistent with what is suggested for laboratory reagent strip lactate testing (25), which establishes a range of 15 to 50 microliters of blood in heparinized capillary pipette, therefore 20 microliters of serum content would correspond to one drop of capillary blood and would be within the praxis of other devices.

Results. 11 bioelectric signals of the reaction between CRP and MPC + SiO2 were possible to consider, and were unidimensional, with function characteristics of a simple variable (time), defined by a deterministic analog (continuous time) signal (23), as shown in Figures 1 to 11, with its integral size calculated as shown in table 1.

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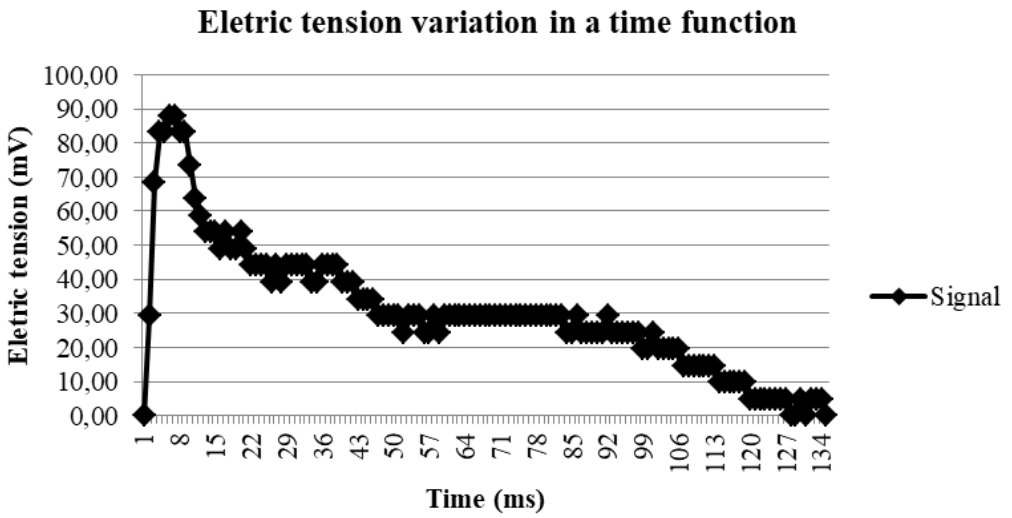

Figure 1. Bioelectric signal obtained from CRP interaction with biomaterial (MPC + SiO2) in the reagent strip.

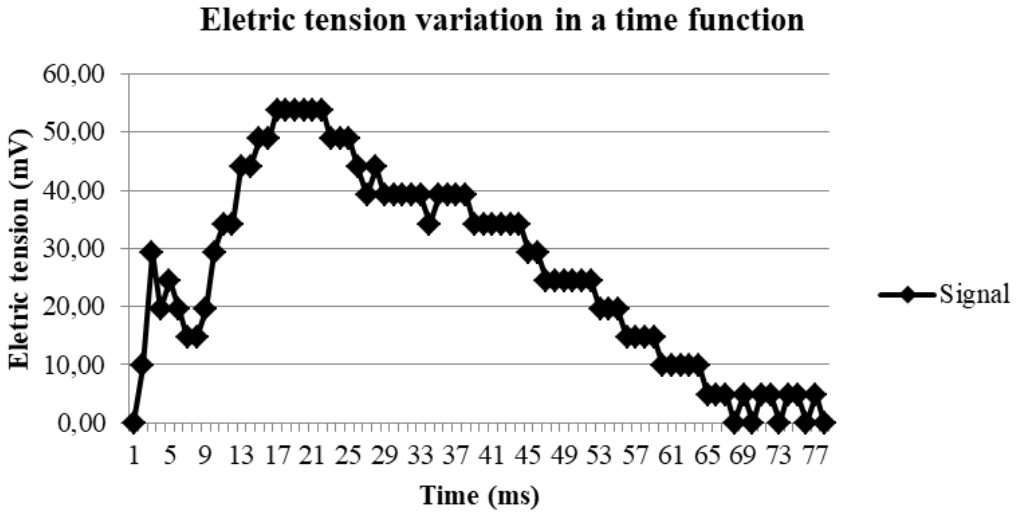

Figure 2. Bioelectric signal number 2 obtained from CRP interaction with biomaterial in the reagent strip.

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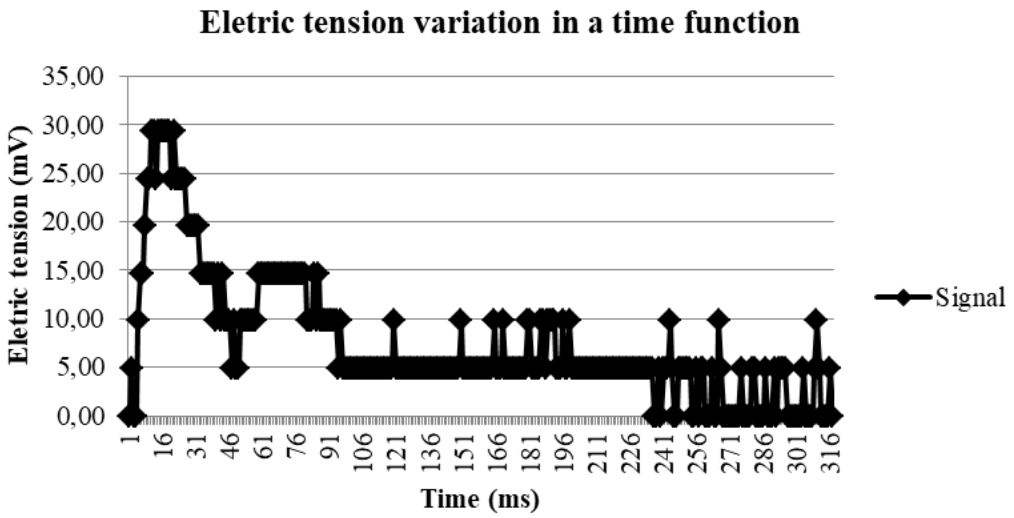

Figure 3. Bioelectric signal number 3 obtained from CRP interaction with biomaterial in the reagent strip.

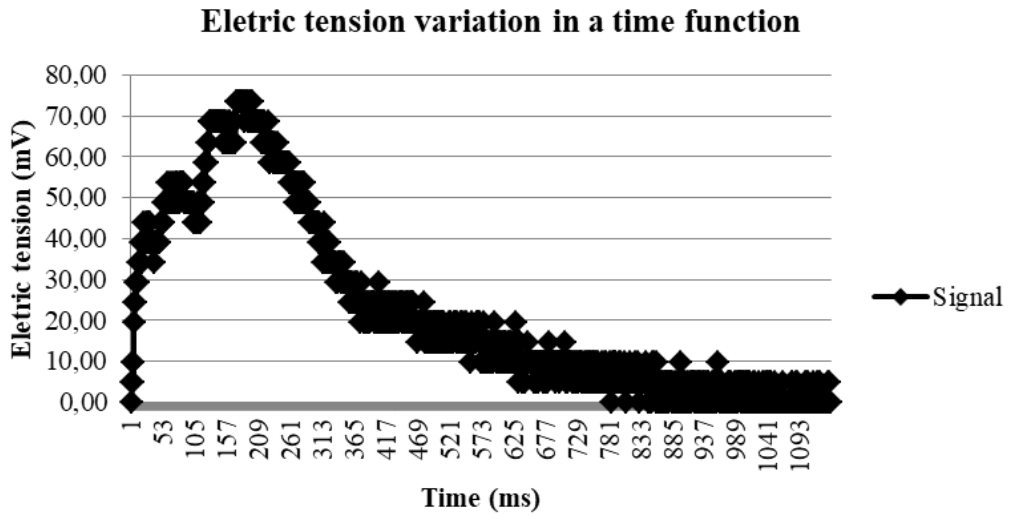

Figure 4. Bioelectric signal number 4 obtained from CRP interaction with biomaterial in the reagent strip.

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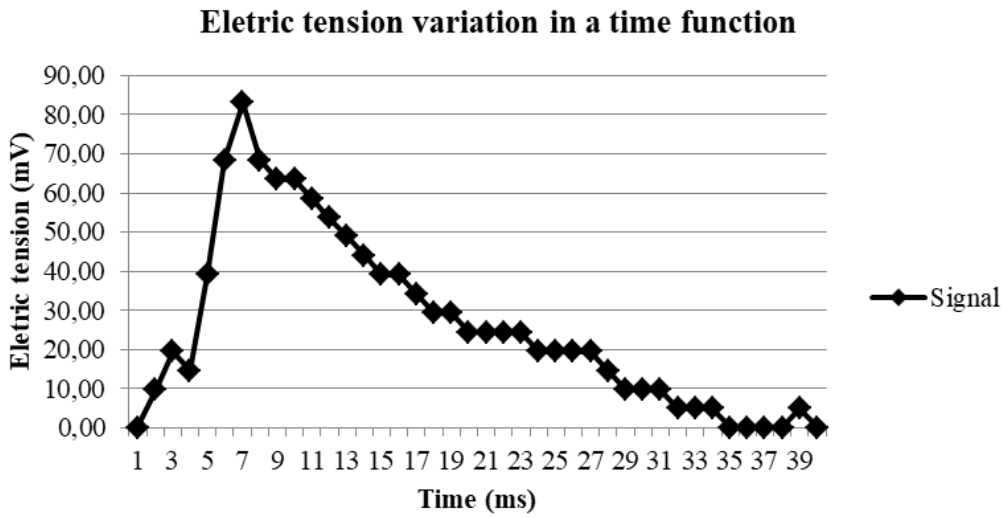

Figure 5. Bioelectric signal number 5 obtained from CRP interaction with biomaterial in the reagent strip.

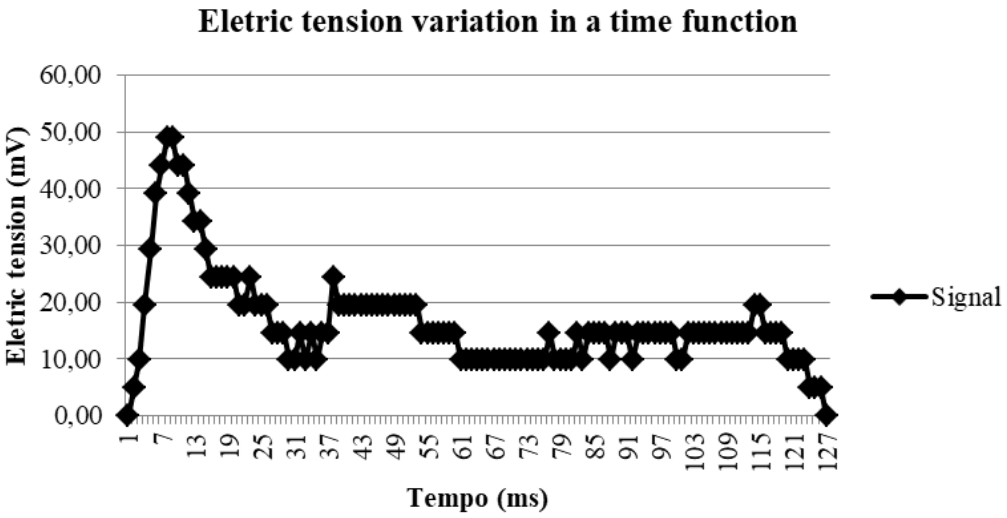

Figure 6. Bioelectric signal number 6 obtained from CRP interaction with biomaterial in the reagent strip.

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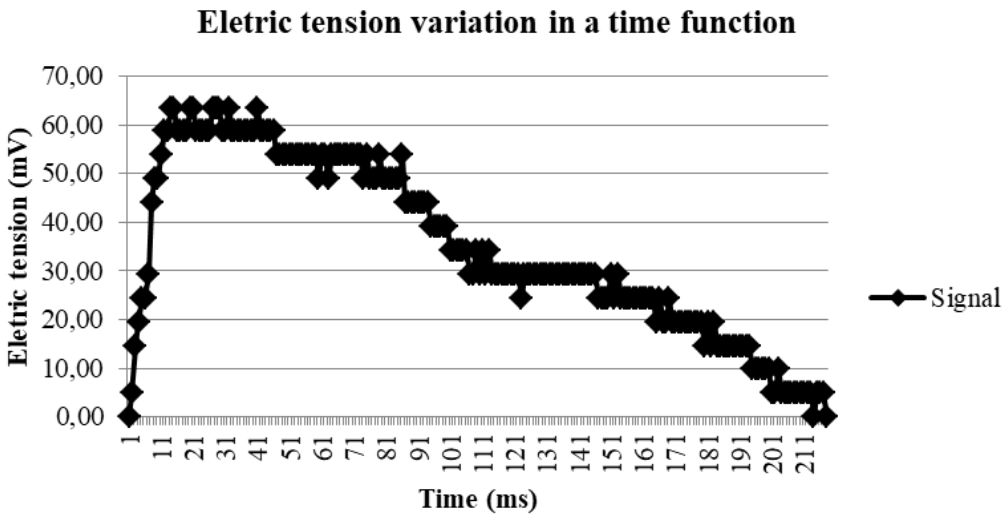

Figure 7. Bioelectric signal number 7 obtained from CRP interaction with biomaterial in the reagent strip.

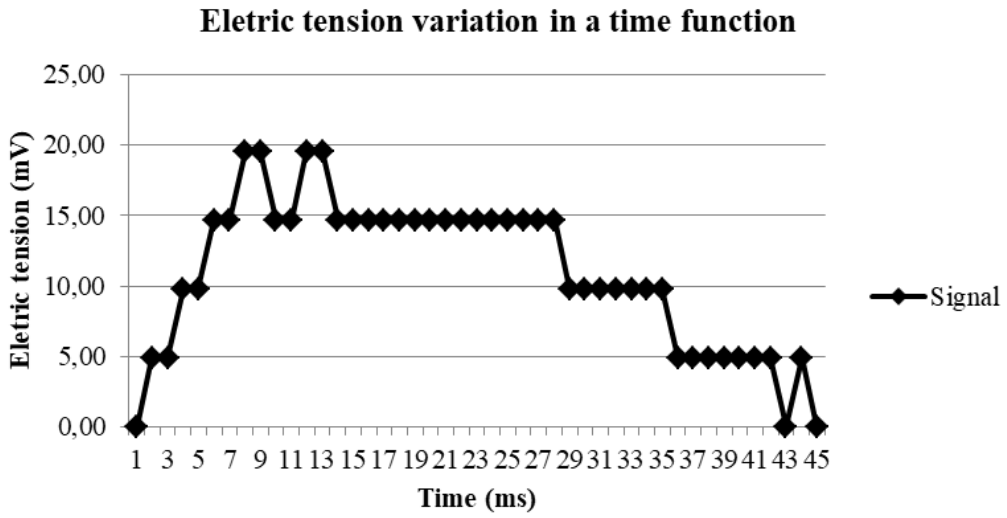

Figure 8. Bioelectric signal number 8 obtained from CRP interaction with biomaterial in the reagent strip.

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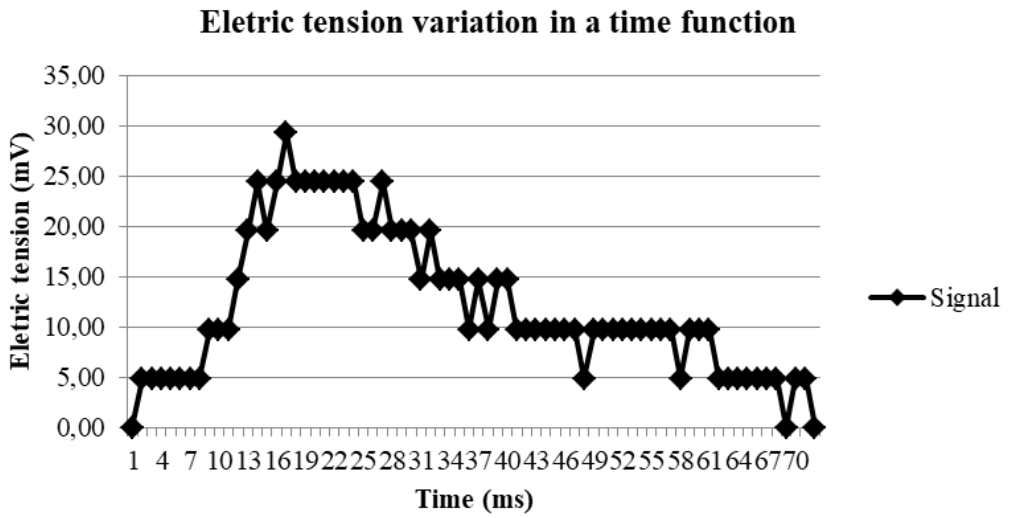

Figure 9. Bioelectric signal number 9 obtained from CRP interaction with biomaterial in the reagent strip.

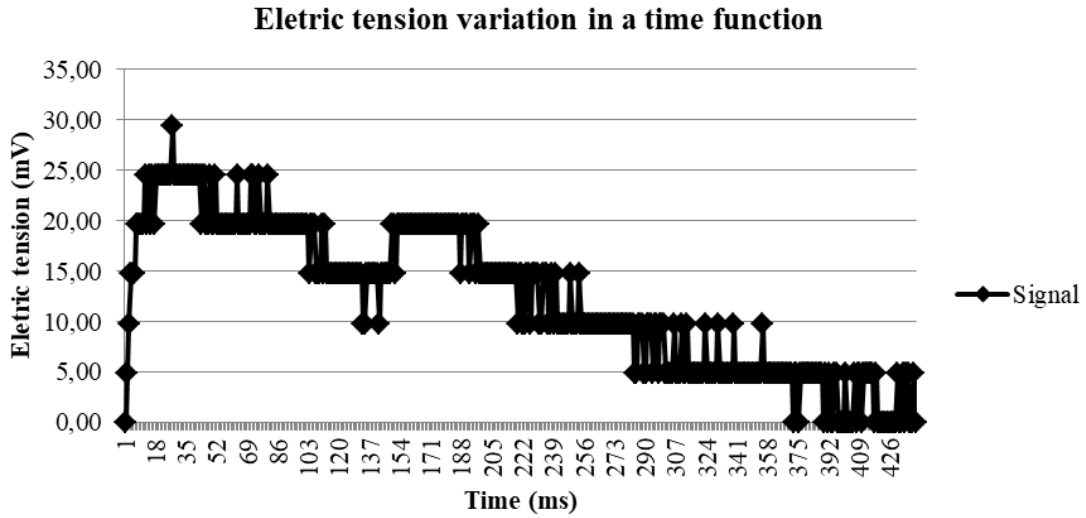

Figure 10. Bioelectric signal number 10 obtained from CRP interaction with biomaterial in the reagent strip.

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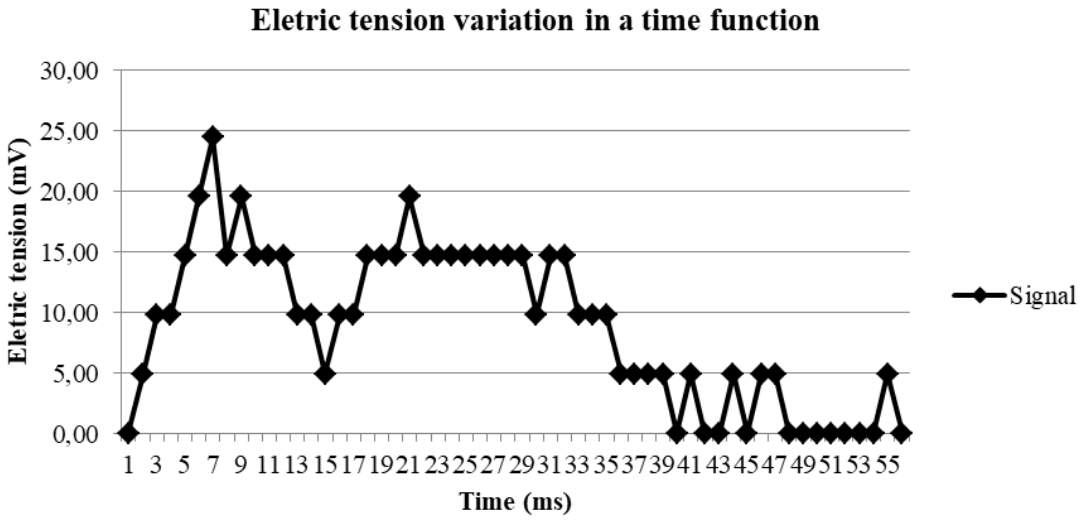

Figure 11. Bioelectric signal number 11 obtained from CRP interaction with biomaterial in the reagent strip.

Table 1. Signals integral sizes (mV).

SIGNALS NUMBERS	SIGNALS INTEGRAL SIZES (mV)	SIGNALS PEAK ELETRIC TENSION (mV)
1	411,35	87,98
2	2038,12	53,76
3	2355,82	29,33
4	25708,70	73,31
5	1045,94	83,09
6	2082,11	48,88
7	7600,20	63,54
8	493,65	19,55
9	869,99	29,33
10	5449,66	29,33
11	498,53	24,44

This signal is a demonstration of feasibility for CRP detection by bioelectrical device, and in pandemic moment of COVID-19, as already mentioned, the CRP levels in patients are elevated to critical values (2-10). This studies suggest the CRP levels monitoring is a procedure for patients care, to help the health professionals in critical decisions, as medical administration, or others interventions.

On the other hand, CRP analysis is expensive and the results are length, because the procedure involves a venous blood sample collection and special transport to a clinical analysis by immunoturbidimetry.

Our experiment is a start development to in the future possible create an electronic device to use a reagent strip to CRP quantification in real time, to application in COVID-19 patients care, and to other applications in inflammatory analysis.

Discussion. Park et al. (26) developed a biomaterial consisting of the combination of MPC with n-butyl methacrylate (BMA) and p-nitrophenyl oxycarbonyl polyethylene glycol methacrylate (MEOMP), thus forming PMBN to emulsify the surface under polar groups of phospholipids, thereby creating a material with polymeric MPC nanoparticles (MPC-PNP). This experiment resulted in the detection of CRP by immobilization of anti-CRP in MPC-PNP, and CRP was detected by scanner electronic microscope.

Despite the authors' successes in applying a sophisticated method for immobilizing CRP and its identification by microscopy, this process is not practical for application in real-time CRP monitoring of patients with COVID-19, as proposed by our group.

The study by Kurosawa et al. (27) used cysteamine hydrochloride, glutaraldehyde and glycine to fix CRP specific antibody in three different preparations (anti-CRP-IgG, anti-CRP F (ab')2-IgG and anti-CRP Fab-IgG), whether or not containing MPC on a quartz crystal microscale (QCM) surface, an instrument that uses a quartz crystal wave oscillator, and upon varying the signal frequency influenced by the deposit of something to be weighed, it is possible to weigh the mass of such deposited material. The results showed high detection of CRP in the preparation with anti-CRP F(ab')2-IgG containing MPC.

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This is an interesting procedure, with the use of immunoglobulins, but it would be difficult to manufacture a reagent strip with this method, as it is necessary to consider the rapid half-life of immunoglobulins.

The study by Kitayama and Takeushi (28) used the visible ultraviolet spectrophotometry method to identify changes in light absorption (color change) caused by the presence of CRP in a container containing gold-conjugated MPC nanoparticles. This variation was calculated by the formula Δ (A/D) / (A/D)0, where A corresponds to the integral of the gold-conjugated MPC spectrum between 550 and 700 nm, D corresponds to the spectrum between 490 and 540 nm. Data showed that the method provided good CRP detection.

This study presents interesting results for detection and CRP, but by another means, spectrophotometry, while our study proposes this detection by means of a bioelectric signal, which would be more practical to develop a CRP measuring equipment along the lines designed by our group for assist patients with COVID-19.

In a study by Matsuura et al (29), a polymer containing phosphorylcholine (poly (2-methacryloyloxyethyl phosphorylcholine - PMPC) was used to bind and react with CRP. This polymer was embedded in a hybrid (biological/chemical) plasmid chip with a layer of approximately 4.4 nm in thickness and 0.7 grams per cm-3 prepared by thin metal film coating resulting in a fluorescence sensing structure (nanotechnology). This form of detection differs from conventional immuno-sensing chips that use immobilized antibodies to capture target analytes.

After CRP was captured by PMPC on the chip, the following were added: 1) biotinylated anti-CRP antibody (biotinylation - addition of biotin molecules (vitamin B7) for protein marking), and 2) streptavidin (protein, purified on bacterium Streptomyces, with high affinity to biotin) marked with fluorescence. Cy5-anti-CRP was also synthesized by coupling reaction between the side chain amino groups of lysine residues in anti-CRP and Cy5-NHS (fluorescent bioconjugate ester).

These preparations were performed so that the captured CRP could be detected by the fluorescence intensity emitted by the reaction between CRP and the phosphorylcholine contained

in the PMPC. Thus, it was possible to analyze 2 forms of CRP detection using the PMPC with embedded plasmid chip, one containing biotin-anti-CRP/Cy5-streptavidin, and another containing Cy5-anti-CRP.

The results showed fluorescent activity in the presence of 1 nM of CRP in both CRP detection methods using PMPC, but with higher fluorescence in the Cy5-anti-CRP method. However, the authors found the condition they used or preparation with biotin-anti-CRP/Cy5-streptavidin to be more interesting, as it showed less nonspecific binding when compared to Cy5-anti-CRP, as it showed a very large statistical error. It is also worth mentioning that the biotin-anti-CRP/Cy5-streptavidin method showed a coefficient of determination proportional to the CRP concentration (r2=0.957).

Similar to the previous study, this one also had a sophisticated method for detecting PCR, but also with light emission analysis, which also makes it difficult to produce reagent strips usable with electronic equipment to monitor PCR in real time in patients with COVID-19.

The study by Díaz-Betancor et al. (30) used a more sophisticated method to detect CRP in MPC hydrogel matrix, by fluorescence using surface reader, resulting in good CRP detection.

Eletric conditions in surfaces containing MPC are presented below.

In the study by Xu, Takai and Ishihara (31), protein absorption and cell adhesion of materials containing MPC with the addition of silica (SiO2) and under three different ionic conditions (cationic, neutral and anionic) were verified, and the addition of silica showed to be essential for protein fixation, indicating that MPC in conjunction with SiO2 could possibly be applied to obtain bioelectric signal when CRP interaction occurs, and such fact was adopted in this work.

The ability of MPC to be used in biosensors is reinforced by the study by Lee et al. (32), who evaluated the electrical conductivity of three biomaterials classified as zuiterionic hydrogels, being the MPC among them. At the time, in addition to comparing the biomaterials, a solution with electrolytes was also used as a reference for these analyses, even with different concentrations. The findings of this study showed good ionic conductivity of the zuiterionic hydrogel prepared with MPC.

This condition make surfaces with MPC as dipoles, something how could be applied in electronic device to capture bioelectric signal of reagent strip, and it was used in us study.

Biocompatibility studies can be applied to biosensors, especially on their interaction on the biomaterial surface and surrounding tissues (33, 34). Its application in supervisory control systems can lead to the creation of cyber-physical systems integrated with interconnected health networks (35, 36).

Finally, the study by Pinyorospathum et al. (37) developed a Whatman-type No. 1 filter paper filter device containing thiol-terminated MPC (MPC-SH) and gold nanoparticle electrodes to detect CRP, the detection of which was measured by electrical current using differential pulse voltammetry. It is noteworthy that the gold electrodes were printed on a wax printer on the paper base, facilitating even the deposition and fixation of the MPC-SH in this apparatus. Another aspect is the existence of two side tabs on the device, one of which was used to deposit calcium and then to deposit the sample with 100 microliters of CRP, and the other was used to deposit potassium ferrocyanide (K3Fe(CN)6) and then deposit potassium nitrate (KNO3). The result showed variation of the electrical signal in the presence of CRP. This whole analysis process lasted an hour and a half, which indicates that the method presents important advance, but needs to be improved so that it can be applied in the technological market, as in the manufacture of electronic devices that use strips to quantify CRP in real time.

Clearly this study was the one that came closest to something applied by our group, but with more sophistication and better resources, which could be a guiding model to improve, with the future result of developing an electronic equipment that quantifies CRP in time in patients with COVID-19, by taking reagents.

Conclusion. Based on the data presented, it is concluded that it is possible to detect the presence of CRP by bioelectric signal, and that the use of MPC is promising for future application in portable electronic device with uses of reagent strips, to application in COVID-19 patients monitoring, and to others populations applications. However, it is necessary to improve the technological development to reach this objective, and our group is currently working on it.

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