# DETERMINATION OF p53 FROM WHOLE BLOOD SAMPLES USING AN ELECTROCHEMICAL SENSOR BASED ON GRAPHENE DECORATED WITH N AND S

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*Tumor protein p53 is used as biomarker for gastric cancer diagnostics. An electrochemical sensor was proposed for its assay in whole blood samples. The electrochemical sensor was design by impregnating a graphene decorated with N and S with 2,3,7,8,12,13,17,18-octaethyl-21H,23H-porphirine manganese (III) chloride solution ($10^{-3}$ mol $L^{-1}$). Differential pulse voltammetry method was used for all measurements performed. The selectivity of the sensor was checked against possible interfering species from whole blood samples. Very good recoveries of p53 were obtained when the electrochemical sensor was used for the assay of the concentration of p53 in whole blood samples.*

**Keywords**: p53, electrochemical sensor, porphyrin, graphene.

## 1. Introduction

Cancer is a high mortality disease in modern society, and it's the cause of a high rate of all deaths globally [1]. Early-stage monitoring of cancer biomarkers is especially important for providing vital information of diagnostics and efficient and time-saving therapy. The protein p53 is a well-known tumor suppressor that plays a vital role in the repairing of DNA, apoptosis and cell proliferation [2].

Consequently, the accurate detection of cancer biomarker p53 protein is very important for the early diagnosis and efficient therapy of cancer. Due to the fact that, in more than half of the cases of cancers, the p53 protein suffers mutations, it was found that in the human sera the level of the protein is even low compared to the normal levels [5], and it was highly desirable to develop a sensitive, reliable and selective analytical technique for detecting p53 level.

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For detection of p53 to date, best performances were achieved using surface plasmon resonance (SPR) [6], and enzyme-linked immunosorbent assays (ELISA) [7].

Although the classical methods have their advantages, the new methods have improved characteristics, such as short-response, low-costs of reagents, and easy to use of equipment. Because they have so many advantages over traditional methods, sensors in general are a good alternative for quantitative measurements. The components used in a sensor's construction determine how effective it is. High sensitivity in detecting the analyte in extremely low concentrations and also good selectivity toward other species that are present in the biological sample constitute a highly significant need, particularly for sensor materials. According to certain investigations, porphyrins [8] and graphene [9] can be used to make sensors that can detect cancer biomarkers by acting as sensing materials [10]. Positive outcomes were attained in the lab of Prof. Raluca van Staden, including low limits of determination, high sensitivities, and sensors that covered a variety of patients and cancer biomarkers [11-13].

Carbon-based compounds have several applications in biomedicine. Graphene and its derivative have increased the use of carbon-based compounds in the biological area because of their unique structure and capabilities [14]. Graphene, the father of all graphitic forms of carbon, is a crystalline substance comprised of sp2 hybridized carbon atoms organized in a honeycomb-like hexagonal arrangement. These materials' ability to interact with other molecules via a number of processes, some of which may be physical [15] and chemical [16] processes, is one of its main advantages.

Doping with heteroatoms has been shown to considerably enhance the catalytic activity of carbonaceous materials and change their electronic properties through an increase in active sites and the modification of electronic states [17-19]. Particularly nitrogen doping has the ability to significantly impact the enhancement of electrode performance [20,21]. The performance of co-doping by a number of elements with different electronegativity is further enhanced due to the substantial number of heteroatoms functioning as active sites and the positive synergistic effect of heteroatom co-doping [22].

Graphene, which is made up of 2D single-atom layers and has tremendous electrical conductivity, excellent flexibility, and a sizable specific surface area, is the most promising electrode material [9,13]. The overlapping agglomeration between the neighboring sheets would, however, occur as a result of the - interaction and van der Waals interactions between the graphene crystal surfaces, drastically reducing the effective surface area and leaving the material with a constrained double layer specific capacitance [23]. Numerous methods have been developed to address the agglomeration problem and improve the properties of

graphene, including chemical doping, metal modification, and combinations with other substances [24-27].

There are many porphyrin derivatives in nature, and they have crucial biological roles in things like oxygen transport, photosynthesis, and catalysis. Porphyrin derivatives have distinctive photophysical and electrochemical characteristics because of their intrinsic -conjugated structure. Porphyrin derivatives interact noncovalently with a variety of protein molecules in biological systems [28]. For instance, hemoglobin, which in most vertebrates' transports oxygen, is made up of four subunits of a globular protein with a prosthetic group made of an iron porphyrin derivative. Furthermore, the essential chromophores in light-harvesting systems for photosynthesis in plants and algae are noncovalently organized derivatives of porphyrins. The diversity of porphyrin derivatives' functional properties is what gives rise to these crucial biological activities. Porphyrins in particular make great host molecules because they can form coordination complexes with a variety of metal ions to provide the porphyrin unit additional functionality including redox activity and more ligand binding at the central metal ion [29,30]. Porphyrins' flat and symmetrical molecular structures make them useful building blocks for functional supramolecular complexes, and their good photophysical characteristics are frequently used to create bioactive functional materials.

In this paper we proposed an electrochemical sensor based on graphene decorated with N and S, modified with 2,3,7,8,12,13,17,18-octaethyl-21H,23H-porphirine manganese (III) chloride solution (Fig.1) for the assay of p53 in whole blood samples.

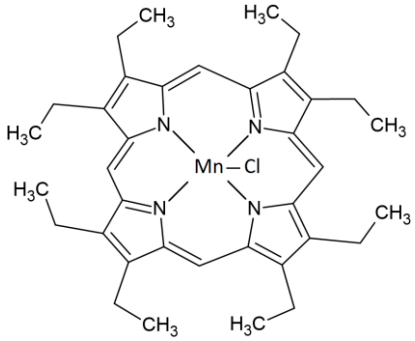


Fig. 1. The chemical structure of 2,3,7,8,12,13,17,18-octaethyl-21H,23H-porphirine manganese (III) chloride

## 2. Experimental

### *2.1 Materials and reagents*

Tumor suppressor p53, monosodium phosphate and disodium phosphate were purchased from Sigma Aldrich. Paraffin oil ($d_{420}$, 0.86 g x $cm^{-1}$) was purchased from Fluka. Monosodium phosphate and disodium phosphate were

used for preparation of phosphate buffer solution (0.1 mol/L, pH=7.5). Deionized water obtained from a Millipore Direct-Q3 System was used for the preparation of all solutions. Standard solution of P53 was prepared in PBS pH=7.5 to a concentration of 7.15 µg/mL. Serial dilution method was used for the preparation of solutions of different concentration for p53.

p53 Human ELISA Kit was purchased from Thermo Fisher Scientific. The ready-to-use ELISA kit (enzyme-linked immunosorbent assay) was used for determining whether the p53 is present in different samples (human serum or cell culture medium). In order to identify p53 antigen targets in samples, the kit's ELISA analytical biochemical method relies on p53 antibody-p53 antigen interactions (immunosorbency). It also uses an HRP colorimetric detection system.

### *2.2. Apparatus*

All measurements of the solutions and samples were performed using an AUTOLAB/PGSTAT 302 (Metrohm, Utrecht, The Netherland), connected to a computer for data acquisition and recording of diagrams. The electrochemical cell consists of the reference electrode, the counter electrode and the working electrode. An Ag/AgCl (0.1 mol $L^{-1}$ KCl) electrode served as a reference electrode and a platinum electrode was used as auxiliary electrode in the setup of the electrochemical cell. All measurements were carried out at 25 °C.

### *2.3. Design of the electrochemical sensor*

The paste used as active side of the electrochemical sensor was obtained by physical mixing 100 µL solution of 2,3,7,8,12,13,17,18-octaethyl-21H,23H-porphirine manganese (III) chloride ($10^{-3}$ mol $L^{-1}$) with 100mg paste (obtained by mixing graphene decorated with N and S with paraffin oil). The modified paste was placed in a non-conducting polymer tube with an internal diameter of 150 µm. Electric contact was made using a silver wire. Between the measurements, the sensor was washed with deionized water and dried. When not in use, the sensor was kept in a dry place at room temperature.

### *2.4. Recommended procedure*

DPV was used for the measurements of each standard solution of known concentration ($1.0 \times 10^{-8}$ µg $mL^{-1}$ – 5.00 µg/mL). The working parameters were as following: scan rate was 90 mV $s^{-1}$, potential range -1 - 0.750 V, and modulation amplitude 50 mV. The equation of calibration ($I=f(Conc._{p53})$) was obtained using linear regression method, and it was used for calculations of unknown concentrations of p53 in whole blood samples.

The human p53 ELISA kit was used to determine the amount of target bound between two antibodies. 96-well plates were pre-coated with an anti-p53

polyclonal antibody. As detection antibodies, biotin conjugated anti-p53 polyclonal antibodies were used. Following that, the standards, test samples, and biotin conjugated detection antibody were added to the wells, and the wells were washed with wash buffer. Unbound conjugates were washed away with wash buffer after the Avidin-Biotin-Peroxidase Complex was added. HRP enzymatic reaction was visualized using TMB substrates. This signal's intensity is proportional to the concentration of target present in the original specimen.

### *2.5. Samples*

Whole blood samples were obtained from the Clinical County Hospital of Targu Mures (Ethics committee approval nr. 75/2015) from 5 different patients diagnosed with gastric cancer. These samples were used for the direct assay of p53 without any pretreatment.

### *2.6. Selectivity studies*

The electrochemical sensor's selectivity was investigated in relation to L, D-glutamine, CEA and L, D-Aspartic Acid. The amperometric selectivity coefficients were calculated using the mixed solutions method to check for interference and determine the selectivity of the proposed electrochemical sensor. Prior to the measurements, the solutions were made using the mixed solution method, taking into account the p53 to interferent molar ratio of 1:10 (mol/mol). The technique was utilized to gain a better grasp of how to use the electrode effectively, making it a suggested way for figuring out amperometric selectivity coefficients.

## 3. Results and discussions

### *3.1. Characteristic response of the proposed electrochemical sensor*

The electro-oxidation of p53 is a complex process given the complexity of the molecule. p53 undergoes post-translational modifications, such as oxidation and covalent modification of cysteines, nitration of tyrosines, acetylation of lysines, phosphorylation of serine/threonine residues.

Differential pulse voltammetry (DPV) technique was used to determine the response characteristics of the proposed electrochemical sensor, when used for the assay of p53. The voltammograms used for the calibration of the proposed sensor were shown in Fig. 2. The half wave potential was recorded at -351 mV. The equation of calibration was:

$$I = 5.79 \times 10^{-7} + 2.89 \times 10^{-8} \times C_{p53} \quad (1)$$

where I is the height of the peak in A, and $C_{p53}$ is the concentration in μg $mL^{-1}$. Fig. 3 shows the calibration curve. The correlation coefficient, r is 0.9532. The sensitivity of the electrochemical sensor is $2.89 \times 10^{-8}$ A/μg $mL^{-1}$. The linear concentration range

was between 8 ng $mL^{-1}$ and 5 μg $mL^{-1}$. The limit of detection was determined as 0.1 ng $mL^{-1}$.

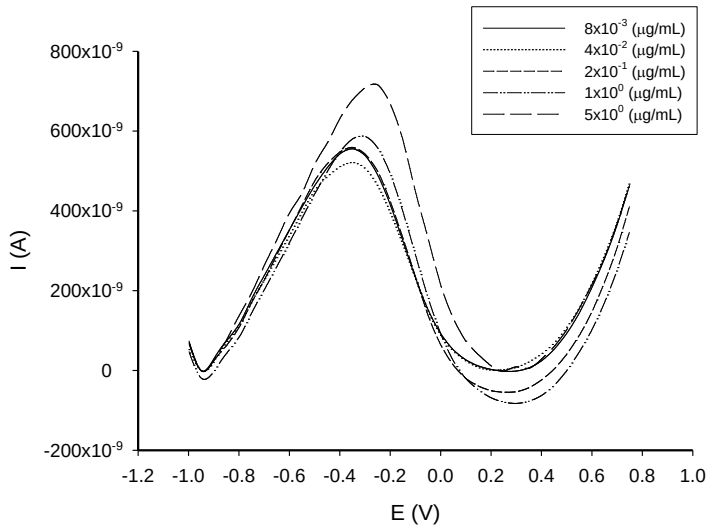


Fig. 2. Differential pulse voltammogram obtained for p53 at different concentrations. The working parameters were as following: scan rate was 90 mV $s^{-1}$, potential range -1 - 0.750 V, and modulation amplitude 50 mV.

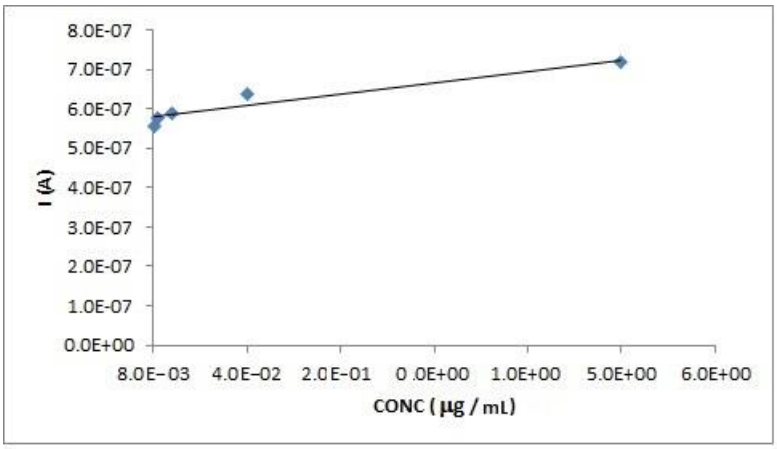


Fig. 3. Calibration graph obtained for p53 using the modified S and N decorated graphene paste based sensor.

The results showed a good value of the sensitivity and a low limit of determination of p53. The proposed sensor covered the range on which p53 can be found on healthy people, as well as for patients presenting stages 1-3 of gastric cancer. Compared to other studies, where in one of the studies a sandwich immunoassay method was utilized, formed between antibody-antigen-streptavidin

complex and graphene material as base platform [31], and the second study, where the researchers used protein cage templated metallic phosphates [32], instead of enzymes, and subsequent stripping voltametric analysis, a better linear concentration range was obtained. All three studies succeeded in obtaining far better results than ELISA. In another study, Yeo and collaborators [33], utilized the protein with the p53 core domain's natural folding structure as a mode. Through host-guest interactions between the lipo-diaza crown and the alkylammonium group on the surface of the antibody, (R)- lipo-diaza-18-crown-6 (lipo-diaza crown) was employed to create a self-assembled monolayer (SAM) on the gold disk electrode for the immobilization of antibody. The results obtained were in the μg concentration range, therefore, compared to our study, the decade of concentration was 10-fold better.

### *3.2. Selectivity of the electrochemical sensor*

Mixed solution method was used as described above for the assessment of the selectivity of the proposed electrochemical sensor. The values obtained for the amperometric selectivity coefficients are shown in Table 1. The amperometric selectivity coefficients were determined using the following equation [34]:

$$Ki,j(amp) = \left(\frac{\Delta It}{\Delta Ii} - 1\right) * \frac{ci}{cj} \quad (2)$$

where $K_{i,j\ (amp)}$ is the amperometric selectivity coefficient, $\Delta I_t = \Delta I_t - \Delta I_b$, where $\Delta I_t$ is the total intensity of the current, $\Delta I_b$ is the intensity of the current recorded for blank solution, $\Delta I_i = \Delta I_i - \Delta I_b$, where $\Delta I_i$ is the intensity of the current registered for main ion, $c_i$ and $c_j$ are the concentrations of the main ion and the interfering ions.

*Table 1*

**Selectivity coefficients obtained for the electrochemical sensor**

| Interferent | Amperometric selectivity coefficient |
|---|---|
| L-aspartic acid | $4.00x10^{-3}$ |
| D-aspartic acid | $4.07x10^{-4}$ |
| L-glutamine | $4.17x10^{-4}$ |
| D-glutamine | $4.68x10^{-4}$ |
| CEA | $1.99x10^{-6}$ |

The values obtained for the amperometric selectivity coefficients shown that L-aspartic acid is slightly interfering in the determination of p53, while D-aspartic acid, L-glutamine, D-glutamine and CEA did not interfere in the assay of p53.

### *3.3. Determination of p53 in whole blood samples*

Five whole blood samples were analyzed using the proposed electrochemical sensor. No pretreatment was done before the measurements. The DPV was used to analyze p53 in blood samples. The cell was filled with the whole blood and the peak height was measured. The results of the differential

pulse voltammetry measurements are shown in Table 2. An example of voltammogram obtained from the measurement using DPV mode for determination of p53 in whole blood sample is illustrated in Fig. 4.

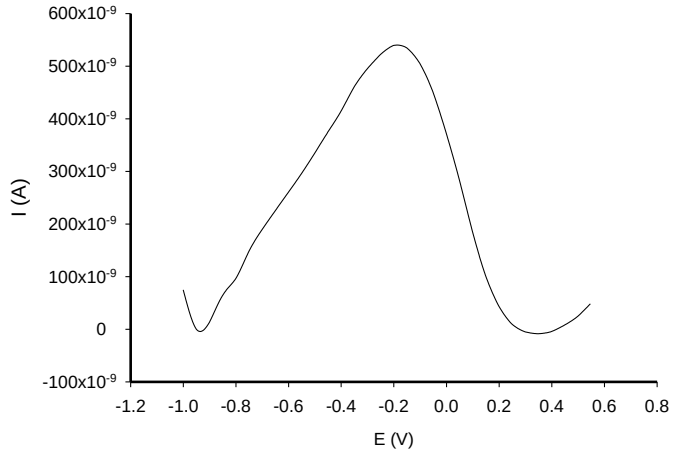


Fig. 4. Example of voltammogram obtained for the assay of p53 in whole blood sample.

*Table 2*

**Determination of p53 in whole blood samples using the electrochemical sensor (scan rate was 90 mV $s^{-1}$, potential range -1 - 0.750 V, and modulation amplitude 50 mV) and ELISA**

| Sample No. | ng $mL^{-1}$, p53 | |
|---|---|---|
| | ELISA | Electrochemical sensor* |
| 1 | 39.87 | 40.00±0.09 |
| 2 | 30.95 | 31.00±0.12 |
| 3 | 36.15 | 36.10±0.13 |
| 4 | 23.07 | 23.00±0.10 |
| 5 | 32.30 | 32.21±0.12 |

*N=10

The results shown a very good correlation between the results obtained using ELISA (a standard method used in the clinical laboratories) and the results obtained using the electrochemical sensors in DPV mode.

## 4. Conclusions

The proposed electrochemical sensor showed very good results for the recovery test which makes it a reliable tool for measuring p53 in whole blood samples. The sensor was highly sensitive and exhibited excellent selectivity for the detection of p53 from blood samples. The advantage of the proposed method versus techniques like ELISA is the fact that it is a simple and easy method performed with low cost, short analysis time, and low limit of quantification, contributing to the diagnosis at a very early stage the gastric cancer.

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