DNA mismatch repair (MMR) is a system for identifying and correcting base errors that may occur during DNA replication and recombination, as well as various types of DNA damage.\(^1,2\) MMR proteins are necessary for the genetic alterations that happen randomly during DNA replication to be identified and fixed. The risk of developing neoplasia is raised by a lack of MMR function (dMMR). The presence of dMMR in tumours can indicate a high probability of Lynch syndrome, an inherited cancer disorder, and can indicate a poor clinical response to several conventional chemotherapies but an elevated chance of response to immunotherapy.\(^2\%–3\%\) of all colorectal cancers (CRCs) are caused by Lynch syndrome (LS), the most common type of inherited predisposition to CRC.\(^2,3\)

Multiple genetic and epigenetic changes, chromosomal instability, microsatellite instability (MSI), and mutations contribute to the pathogenesis of gastric cancer (GC). Although 10% of GCs appear to have a familial tendency, the majority of GCs exhibit chromosomal instability, and half of them can be linked to hereditary germ line mutations.\(^4,5\) Frameshift mutations accumulate in these genes as a result of the failure of mismatch repair (MMR) genes to correct errors that arise during DNA replication in microsatellites, which causes DNA mismatch replication errors and microsatellite instability.\(^6–8\) Microsatellites are units of two or more nucleotides or single nucleotide repetitions in the genome’s DNA sequence that are referred to as microsatellites.\(^9\)

In humans, seven MMR proteins \(\text{mMLH1 homologue 1 (MLH1)}, \text{mMSH2 homologue 3 (MLH3)}, \text{mMSU homologue 2 (MSH2)}, \text{mMSU homologue 3 (MSH3)}, \text{mMSU homologue 6 (MSH6)}, \text{postmeiotic segregation increased 1 (PMS1)}\) and postmeiotic segregation increased 2 (PMS2) work coordinately in sequential steps to initiate repair of DNA mismatches.\(^10\) MLH1, MSH2, MSH6, and PMS2 are the main MMR proteins involved in the process of repairing DNA mismatch replication.\(^6,7,9,11\) Due to a flaw in the DNA MMR system, MSI is a very helpful technique for the identification of families affected by hereditary nonpolyposis colorectal cancer (HNPPC) or Lynch syndrome.\(^9,11,12\) A lack of MLH1 (9.8%), MSH2, MSH6, or PMS2 (all 1.5%) is the most common cause of the DNA mismatch repair deficiency that affects 13% of colorectal tumors.\(^13\) In the heterodimer structure of these four proteins, MLH1 connects with PMS2 and MSH2 with MSH6. While the MSH-2/MSH6 coupling functions as an endonuclease, the MLH1/PMS2 pairing recognizes mismatched nucleotide base pairs and starts repair.\(^14\) The MMR genes MLH1, MSH2, MSH6, and PMS2, which correspond to these proteins, are responsible for encoding them.

Oncogenes are what are known as the gene category that contains the Kirsten rat sarcoma virus (KRAS) gene.\(^15\) Oncogenes have the ability to transform normal cells into malignant cells if they are altered in some way. It is responsible for the encoding of its homologous protein, which plays a role in the division of cells, the differentiation of cells, and the death of cells (apoptosis).\(^16\) In colorectal malignancies, KRAS mutations are detected in MSI and microsatellite stable (MSS) tumours, while in GC, KRAS mutations are mainly observed in the MSI subtype.\(^17\) BRAF testing, immunohistochemistry, or polymerase chain reaction (PCR) can be used to identify mismatch repair proteins defects.\(^18\) Alternatively, KRAS protein can be identified through Sanger sequencing.\(^19\) single nucleotide primer extension assays,\(^20\) pyrosequencing,\(^21\) but also with analytical techniques, such as differential pulse voltammetry (DPV),\(^22\) electrochemiluminescence\(^23\) or chronoamperometry.\(^24\)

Stochastic methods were employed in biomedical analysis due to their benefits over standard methods of analysis: they are ease of use (no sample treatment was required), highly sensitivity, cost effective, they have the ability to detect several analytes, they are highly selectivity, and low detection limits are recorded.\(^25\) It is well-known that stochastic sensors can conduct accurate qualitative and quantitative analyses.\(^26–27\) In the past, they have been used for biomedical analysis since they may be applied to screening tests for any biological fluid, including saliva, urine, whole blood, and serum samples.\(^28–31\) Because the results of the study are unaffected by the composition of the matrix from which the biomarker is determined, they can be evaluated for use in screening tests for the recognition of molecular patterns of clinical relevance.\(^32\) The advancement of nanostructured materials is directly tied to the development of such sensors. Diamond and graphene materials have been demonstrated to be effective sensor design matrices.\(^33,34\) Their modification with nanostructured materials like maltodextrins and porphyrins would enable stochastic detection via the development of pores/channels, which will facilitate the detection of biological molecules. The electrodes made of carbon paste are quite compatible with chemical alterations. Utilizing different electron transfer mediators, such as nanomaterials, metal complexes,\(^35\) zeolites,\(^36\) and organic molecules,\(^37\) they can be altered. Due to their peculiar physical and chemical characteristics, metal nanostructures such as those made of cobalt, nickel, iron, and other metals have recently gained interest in...
the field of electroanalysis. When compared to bare electrodes, metal nanostructure-modified electrodes often demonstrate strong electrocatalytic activity toward substances with slow redox processes.

To our knowledge, no electrochemical method was used to date for the assay or simultaneous assay of the MLH1, MSH2, MSH6, PMS2 and KRAS biomarkers. Due to the importance of simultaneous molecular recognition and quantification of these biomarkers, two stochastic sensor based on graphene doped with nitrogen and boron, and modified further with a complex between protoporphyrin IX and cobalt were designed, characterized and validated using biological samples, such as whole blood, urine, saliva and tumoral tissue.

Materials and Methods

Chemicals.—All compounds utilized in this investigation were of the analytical grade. Protoporphyrin IX-Coal Chloride, MLH1, MSH2, MSH6, PMS2, and KRAS were acquired from Sigma Aldrich (Milwaukee, USA). We bought parafﬁn from Fluka (Buchs, Switzerland).

All solutions were created using distilled water that was provided by a Millipore Direct Q-3 System at various concentrations (for MLH1: 32.00 g ml\(^{-1}\) to 3.20 \times 10^{-10} g ml\(^{-1}\), for MSH2: 10.00 g ml\(^{-1}\) to 1.00 \times 10^{-9} g ml\(^{-1}\), for MSH6: 23.00 g ml\(^{-1}\) to 2.30 \times 10^{-9} g ml\(^{-1}\), for PMS2: 27.00 \mu g ml\(^{-1}\) to 2.70 \times 10^{-9} \mu g ml\(^{-1}\) and for KRAS: 22.00 \mu g ml\(^{-1}\) to 2.20 \times 10^{-9} \mu g ml\(^{-1}\)). The analytes solutions were made using phosphate buffer saline (pH 7.40). All solutions were stored in a freezer at −20 °C when not in use.

Graphene oxide (GO) was synthesized using a modiﬁed Hummers method. Urea was purchased from Alfa-Aesar (Germany) and boric acid from Adra Chim (Romania).

The ﬁrst graphene sample (NBGr-1) was prepared as following described: 300 mg GO dispersed in 60 ml H\(_2\)O were mixed by sonication (1 h) with 300 mg urea and 300 mg boric acid. The sample was then dried by lyophilization and subjected to thermal treatment: 30 min at 700 °C under Ar atmosphere. According to XPS analysis, the sample contains heteroatoms: nitrogen (10.8 at%) and boron (2.6 at%).

The second graphene sample (NBGr-2) was prepared as following described: 700 mg GO were dispersed in 120 ml H\(_2\)O by sonication (1 h). After that, 1000 mg urea and 1000 mg H\(_3\)BO\(_3\) were added and the suspension was stirred for 1 h at room temperature. The obtained mixture was then poured into a 250 ml autoclave and placed in the oven at 120 °C for 12 h. After cooling to room temperature, the sample was ﬁltered, washed with distilled water and dried by lyophilization. Finally, the sample was thermally treated at 400 °C for 30 min. According to XPS analysis, the sample contains heteroatoms, nitrogen (9.3 at%) and boron (2.4 at%).

** Instruments.**—To test the solutions and biological samples, GPES software was loaded on a personal computer and connected to an AUTOLAB/PGSTAT 302 potentiostat/galvanostat (Methrom, Utrecht, The Netherlands). A three-electrode electrochemical cell was also employed. The three-electrode system’s working electrodes are the suggested stochastic sensors. The counter electrode, which is a platinum wire, serves as the electrode for comparison with the reference electrode, the Ag/AgCl electrode.

The samples were morphologically and structurally characterized using scanning electron microscopy (SU-8230 STEM system, Hitachi, Japan), X-ray powder diffraction (Bruker D8 Advance Diffractometer) and X-ray photoelectron spectroscopy (SPECs spectrometer equipped with a dual anode X-ray source AlMg, a PHOIBOS 150 2DCCD hemispherical energy analyzed and a multichanneltron detector).

**The stochastic sensors’ design.**—The two synthesized nitrogen-boron doped powders (NBGr-1 and NBGr-2) were used to make the two stochastic sensors. For both sensors, 0.05 g of graphene and parafﬁn oil were mixed to make a homogenous paste. To create the necessary channels and amplify the signal generated by the stochastic sensors a solution of PIXCoCl was used. By physically mixing the paste with a solution of PIXCoCl (10^{-3} mol l\(^{-1}\)) in a ratio of 1:1 (m v^{-1}, mg ml\(^{-1}\)), the paste was further modiﬁed using the above-mentioned solution. The pastes’ contact with the electrochemical cell’s external circuit was made by a silver wire. Each modiﬁed paste was placed into 5 mm long with a 150 μm inner diameter non-conducting plastic tubes. Whether measuring solutions or biological samples, the stochastic microsensors were washed with de-ionized water and dried after each measurement. These sensors are stored in a dry area while not in use.

**Stochastic mode.**—For the stochastic method, a chrononaamperometric approach was selected with a constant voltage (125 mV vs Ag/AgCl) applied. This method was used to qualitatively and quantitatively assess the expression of MLH1, MSH2, MSH6, PMS2, and KRAS in whole blood, tumoral tissue, urine, and saliva from conﬁrmed patients with stomach or colon cancer. Signatures of the five analytes (t_{off} values) were used for their identiﬁcation in the diagrams while the t_{on} corresponding values found in the diagrams recorded for the biological samples were used for the qualitative determination of the analytes accordingly with the equation \(1/t_{on} = a + b \times \text{Con}_{\text{ConfPMS2/MSH2/MLH1/MSH6/KRAS}}\). The two parameters, t_{off} and t_{on} are qualitative and quantitative parameters, the ﬁrst one regarding the signatures of the analytes, and the latter, is referred to as the duration of equilibrium necessary for the redox reactions that occur inside the wall channels as well as the interaction of the analytes with those channels.

![Figure 1](image-url)  
**Figure 1.** Representative SEM image of NBGr-1 sample (a); the XRD pattern and corresponding structural parameters (b).
Biological samples.—In this study, patients who had previously been diagnosed with gastric and colon cancer had samples of their blood, tumoral tissue, saliva, and urine taken from them. These were then analyzed. Before the collection of the samples, not a single one of the patients was undergoing treatment for cancer of the colon or stomach. These samples were obtained from the Hospital of Targu-Mures, which was granted permission to conduct the research by the Ethics Committee with the number 75/2015. The biological samples that were being evaluated did not need to go through any processing steps before being measured. For the purpose of determining the unknown MSH2, KRAS, PMS2, MSH6, and MLH1 concentrations in the biological samples, the stochastic approach described above was used.

Results and Discussion

Morphological and structural characterization of NB-doped graphene samples.—Figure 1a shows a typical SEM image of the first sample, NBGr-1. It can be seen that crumpled and wrinkled nanosheets are randomly arranged and overlapped with each other. The wave-like morphology of the sample may be the result of the heteroatoms or defects present in the graphene plane.

The XRD pattern recorded in the 2θ range of 5°–80° shows the well-defined (002) diffraction peak, at 2θ ≈ 25° (Fig. 1b). The peak is slightly asymmetric and was deconvoluted in two theoretical Gaussians peaks, at 2θ = 23.4° and 2θ = 26°, respectively. Bragg’s equation was used for estimating the distance, d, between two adjacent graphene layers (see the inset table). In addition, Scherrer’s equation was applied for estimating the average crystallite size, D, of graphene (inset table). Hence, one can see that NBGr-1 sample consists of 3–9 graphene layers in a stacking nanostructure with an average crystallite size of about 1–3 nm and an interlayer distance varying from 0.34 to 0.38 nm. Taking into account the ratio of each deconvoluted peak area to the total area of the diffractogram, the proportion of 3 and 9 layer graphene within the sample was determined to be 45% and 55%, respectively.

The morphology and structure of the second sample, NBGr-2, were also investigated and the results are shown in Fig. 2. As displayed in Fig. 2a, the sample exhibits the typical wave-like morphology of graphene, being in excellent agreement with the XRD results (Fig. 2b). In this case, the well-defined (002) diffraction peak at 2θ ≈ 25° is highly asymmetric and it was deconvoluted in two theoretical Gaussians peaks, at 2θ = 22° and 2θ = 25.6°, respectively. The first deconvoluted peak (2θ = 22°) appears due to the reflections of bi-layer graphene crystallites, having an interlayer spacing (d) of about 0.403 nm. The second deconvoluted peak (2θ = 25.6°) appears due to the reflections of six-layer graphene crystallites, being characterized by an interlayer spacing of 0.348 nm. NBGr-2 consists of 77% bi-layer graphene and 23% six-layer graphene (see the inset table). The crystal size of graphene, D, calculated using the Scherrer equation, is between 0.887 and 2.645 nm.

Response characteristics of stochastic microsensors.—The stochastic sensor response was based on channel conductivity $I$ (1): since entering the channel, the analyte blocks the channel and the current drops to zero for a certain time (until the entire molecule enters the channel) the time spent for this step is called the signature of the analyte (found in the diagrams as $t_{off}$); (2) in the second step, the analyte binds the internal channel wall and undergoes a redox process for a certain time, which is correlated with the concentration of the analyte, the time needed for this step is marked in the diagrams as $t_{on}$. Different signatures recorded for the biomarkers (Table I) enable their simultaneous analysis.

The biomarker signature values showed that they might be found simultaneously in the biological sample.

For the assay of PMS2, and MLH1, the stochastic microsensor based on NBGr-1 recorded higher magnitude orders for the sensitivity, whereas the sensor based on NBGr-2 recorded greater sensitivities for the assay of MSH6, and KRAS. When MSH2 was measured using both stochastic microsensors (based on NBGr-1 and NBGr-2), there was no discernible difference in the magnitude orders of sensitivities.

The limits of determination obtained for the assay of PMS2 and MLH1 using the microsensor based on NBGr-1 were lower than those obtained using the microsensor based on NBGr-2. The working concentration ranges are broad and include PMS2, MSH2, MSH6, MLH1, and KRAS concentrations in both healthy individuals and patients with various stages of gastric and colon cancer.

When the parameters of the equation of calibration ($a$ and $b$ values) remained the same as those determined in aqueous solutions of the biomarkers, and the working concentration ranges and limits of determination did not change either, calibrations of PMS2, MSH2, MSH6, MLH1, and KRAS were also performed in biological samples (whole blood, urine, saliva, and tumoral tissue).

Reproducibility and stability of the stochastic sensors.—For each sensor, repeatability studies were conducted. In this regard, the procedure outlined in the sensor design paragraph was used to create 10 of each type of sensor. For KRAS, MLH1, MSH2, PMS2, and
| Stochastic sensors          | Working concentration range (g ml⁻¹) | Calibration equation and r^a | Signature, t_{off} (s) | Sensitivity (s⁻¹/μg ml⁻¹) | Limit of determination g ml⁻¹ |
|-----------------------------|--------------------------------------|-----------------------------|------------------------|--------------------------|-----------------------------|
| PMS-2                       | 2.7 × 10⁻¹⁵–2.7 × 10⁻⁹               | 1/ton = 0.02 + 1.06 × 10⁵C  | 2.2                    | 1.06 × 10⁵               | 2.7 × 10⁻¹⁵                 |
|                             |                                      | R = 0.9999                  |                        |                          |                             |
| MSH-2                       | 2.7 × 10⁻¹³–2.7 × 10⁻⁶               | 1/ton = 0.04 + 22.87C       | 2.4                    | 22.87                    | 2.7 × 10⁻¹³                 |
|                             |                                      | R = 0.9997                  |                        |                          |                             |
| MSH-6                       | 1.0 × 10⁻¹³–1.0 × 10⁻⁵               | 1/ton = 0.04 + 7.67 × 10²C  | 2.5                    | 7.67 × 10²               | 1.0 × 10⁻¹³                 |
|                             |                                      | R = 0.9993                  |                        |                          |                             |
| MLH-1                       | 2.3 × 10⁻¹⁴–2.7 × 10⁻⁷               | 1/ton = 0.02 + 2.25 × 10⁵C  | 3.7                    | 2.25 × 10                | 2.3 × 10⁻¹⁴                 |
|                             |                                      | R = 0.9998                  |                        |                          |                             |
| KRAS                        | 2.2 × 10⁻¹⁴–2.2 × 10⁻⁴               | 1/ton = 0.03 + 1.99C        | 3.4                    | 1.99                     | 2.2 × 10⁻¹⁴                 |
|                             |                                      | R = 0.9999                  |                        |                          |                             |

a) < 1/t_{off} > = s⁻¹; <C >—concentration = μg ml⁻¹; <r >—correlation coefficient.
Table II. Selectivity of the proposed stochastic sensors used for the assay of MLH1, MSH2, MSH6, PMS2, and KRAS.

| Stochastic sensors modified with PIXCoCl and based on | MLH1 signature (s) | MSH2 signature (s) | MSH6 signature (s) | PMS2 signature (s) | KRAS signature (s) | CA19-9 signature (s) | p53 signature (s) | Glutamine signature (s) | CA72-4 signature (s) |
|------------------------------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|---------------------|-------------------|------------------------|----------------------|
| NBGr-1                                                | 3.9                | 2.5                | 3.7                | 2.2                | 3.4                | 0.5                 | 3.0               | 1.7                    | 2.8                  |
| NBGr-2                                                | 3.0                | 4.0                | 4.4                | 2.4                | 3.4                | 0.7                 | 2.6               | 0.7                    | 3.3                  |
Figure 3. Pattern recognition of PMS2, MSH2, MSH6, KRAS, and MLH1 in (a) whole blood samples, (b) saliva, (c) urine and (d) tumoral tissue using stochastic microsensor based on PIXCoCl/NBGr-1.

Figure 4. Pattern recognition of PMS2, MSH2, MSH6, KRAS, and MLH1 in (a) whole blood samples, (b) saliva, (c) urine and (d) tumoral tissue using stochastic microsensor based on PIXCoCl/NBGr-2.
MSH6, the sensitivities of each sensor were determined and compared when immersed in solutions of pH 7.40. The sensitivities for the five analytes had the following RSD (%) values: 0.76% KRAS, 0.52% MLH1, 0.77% MSH2, 0.87% PMS2, and 0.19% MSH6. The repeatability of the sensor design was demonstrated by the sensitivities’ RSD (%) readings.

Figure 5. Determination of PMS2, MSH2, MSH6, KRAS, and MLH1 in (a) whole blood samples, (b) saliva, (c) urine and (d) tumoral tissue using the proposed stochastic sensors based on PIXCoCl/NBGr-1.
Figure 5. (Continued.)
As described in the section “The stochastic sensors” design, 20 sensors of each type were stored in order to evaluate the stability of each sensor. After 30 d, when the complete batch of sensors has been used, the sensitivities of each measurement were recorded for comparison. Each day, a fresh sensor was removed from storage and immersed in solutions containing PMS2, MSH2, MSH6, KRAS, and MLH1 in (a) whole blood samples, (b) saliva, (c) urine and (d) tumoral tissue using the proposed stochastic sensors based on PIXCoCl/NBGr-2.

Figure 6. Determination of PMS2, MSH2, MSH6, KRAS, and MLH1 in (a) whole blood samples, (b) saliva, (c) urine and (d) tumoral tissue using the proposed stochastic sensors based on PIXCoCl/NBGr-2.
and MLH1 at varying concentrations at pH 7.40. The end-of-period results showed high stability of the sensors over time, with 0.11% for PIXCoCl/NBGr-1 and 0.15% for PIXCoCl/NBGr-2 for the change in sensitivity over time.

Selectivity.—The selectivity of the stochastic sensors is given by the differences in the signatures of the biomarkers/analytes from a sample. Different signatures mean there is selectivity. While each biological molecule is unique, and its shape, size, and whether it is
and multiply the value by 100. Results from Table III proved the recovery tests were performed before and after the addition, and % recovery was obtained using the proposed stochastic sensors (Tables SI-IV), and the matching t was read and employed in the stochastic mode as indicated.  

Table III shows the varied toff values for each biomarker/possible interferent using the stochastic sensors based on PIXCoCl/NBGr-1 and based on PIXCoCl/NBGr-2.

### Molecular recognition and quantification of PMS2, MSH2, MSH6, KRAS, and MLH1

Whole blood, gastric tumor tissue, urine, and saliva were collected from individuals with stomach and colon cancer, and the two stochastic sensors were used for rapid screening of the five analytes, PMS2, MSH2, MSH6, KRAS, and MLH1. Each biomarker was first detected in the diagrams (Figs. 3a–3d and 4a–4d) using its signature, and then the matching toff was compared with the added amount (Table III).

The data were subjected to a paired Student t-test, and the level of confidence was 99%. The fact that the calculated t-values for each sample type were less than the theoretical value (4.13) indicates that there is no statistically significant difference between the results obtained using the proposed stochastic sensors (Tables SI–IV), and that disposable stochastic sensors can be relied upon for the molecular identification and quantification of MSH2, KRAS, PMS2, MLH1 and MSH6 in the selected biological samples. Recovery tests were also performed using addition of known amounts of biomarkers to the biological samples. Measurements were performed before and after the addition, and % recovery was calculated by dividing the determined amount to the added amount and multiply the value by 100. Results from Table III proved the high reliability (given by the high values of recovery and low values of %RSD) obtained when the proposed sensors were used for the assay of five biomarkers, PMS2, MSH2, MSH6, KRAS, and MLH1, in biological samples such as urine, saliva, whole blood and tumoral tissue. The proposed stochastic sensor has been shown to be reliable for pattern recognition of the five biomarkers in biological samples from patients found at very early stages, as well as patients found at later stages, of gastric and colon cancer. The main feature of these sensors is their utilization as tools in screening tests for early detection of colon and gastric cancers.

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