MULTIMAL – A point-of-care device for non-invasive multiplexed diagnosis of malaria

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ABSTRACT

A graphene-based electronic biosensor technology was developed to simultaneously detect multiple malaria infections in saliva samples. A fabrication sequence compatible with industrial scale fabrication was developed, enabling the manufacture of more than 700 sensor chips on a wafer, with a high yield. The sensor chips functionalized with probe DNA were characterized using artificial target DNA in simple buffers and showed detection levels at the attomolar range, a level comparable to the lab standard molecular methodologies. Tests using commercial saliva of healthy people spiked with artificial DNA showed response. Further developments in sample interferences and pre-process are on-going.

Keywords: Malaria; Rapid diagnosis test; Graphene field-effect transistor; Biosensor.

1. INTRODUCTION

- Malaria is still one of the deadliest diseases worldwide and its negative impact can be prevented through timely diagnosis and treatment. Light microscopy and rapid diagnostic tests (RDTs) are the main diagnostic tools used in the field for malaria diagnosis whereas polymerase chain reaction (PCR) is mostly used for molecular diagnosis in sophisticated laboratories. These methodologies are time consuming, require skilled specialist/ interpretation and show poor sensitivity for early/ asymptomatic malaria infections [1].

Therefore, alternative rapid and robust diagnostics tools to use in the field are of paramount importance for disease control. Moreover, there is the additional need to discriminate the different malaria infections. Diagnosis of asymptomatic patients which present low-level parasitemia is also a challenge that needs to be addressed. Commercially available RDT are responsible for non-reliable diagnosis when compared to molecular approaches and have the drawback of invasive blood samples use.

- This project aims to develop a multiplex diagnosis device for all malaria species which also allows the identification of asymptomatic patients by using non-invasive samples (saliva) with analytical performance at the same level as standard molecular approaches. The combination of graphene based sensors and DNA enhances its analytical response when compared to the standard DNA detection systems opening the possibility to detect malarial DNA in biological samples (urine, saliva) characterized by their trace levels of DNA. This innovative sensing device can diagnose multiple malaria infections which determines the therapeutics and suitable personalized treatment.

- The sensing device is composed of an electronic reader and a disposable chip. The disposable chip was designed with a layout with 7 distinct regions of 3 sensors each. Each region is with a specific DNA sequence allowing the multiplex detection of all 5 malaria infections and the *Plasmodium* and human control.
  Upon successful fabrication of graphene sensors with multiplex layout, the surface chemistry was optimized allowing the detection of trace amounts of DNA (attomolar), for all the 7 DNA sequences, in controlled conditions. This feature is especially relevant considering the need to detect low amounts of DNA in saliva and biological fluids of asymptomatic patients. Sequential reading of all sensors was possible within less than 5 seconds. Saliva samples with and without treatment were tested allowing DNA detection. Given the

complexity of saliva, their interference compounds are still being evaluated. Meanwhile the correlation between parasites and DNA copies is already being established.

2. STATE OF THE ART

Elimination of Malaria was declared as one of the goals to be achieved until 2030. In order to reduce malaria prevalence, and future elimination, highly accurate diagnostic tools are needed.

The current technologies for rapid diagnostics of malaria do not address the diversity of the infections, can only analyse blood samples where parasites or their metabolites are present in high concentrations, and show poor sensitivity for early malaria infection. Moreover, these tests fail to diagnose asymptomatic patients which is crucial but challenging due to the low concentrations of parasites at that stage.

We address this issue by using a multiplex disposable graphene genosensor device, capable of detecting all Human Plasmodium infection species in a single measurement, enabling a personalized effective treatment.

When compared to the state of the art, our project will: a) increase the accuracy of diagnosis of malaria through the discriminative nature of DNA together with the sensory properties of graphene; b) develop a sensing device with high sensitivity, crucial for the diagnosis of asymptomatic malaria; c) allow for the multiplex diagnosis of all malaria species that infect Humans in a single droplet of unprocessed biological samples; d) take advantage of the high stability of graphene with regard to temperature and humidity, crucial for applications in tropical settings; g) allow a less expensive, faster and user friendly diagnosis of malaria, when compared to standard DNA analysis procedures, without the need for highly trained personnel or data interpretation. Overall, quality and reliability of patient care will be enhanced by the monitorization and tracking of malaria transmission, through discriminative, accurate and affordable diagnosis of malaria infections, which will pave the way for personalized medicine and, ultimately, malaria elimination.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

The project aims to develop a multiplex diagnosis device for all malaria species and asymptomatic patients using non-invasive samples (saliva) with analytical performance at the same level as standard molecular approaches. The combination of graphene based sensors and DNA enhances the response of the typical DNA detection systems opens the possibility to detect malarial DNA in biological samples (urine, saliva) characterized by their low amounts of DNA present. This innovative sensing device can diagnose single or multiple malaria infections which determines the type of therapeutics and suitable personalized treatment. Table 1 shows the comparison of the product developed in this proposal, as compared to products existing on the market.

Tab. 1. Comparison of rapid test technologies for malaria diagnosis.

Products	Characteristics
*Abbott BinaxNow**	HRP2 - AB P. falciparum + mix of P.v./P.o./P.m. < 15 min whole blood (finger-prick)
*Abbott SD Bioline Malaria AG P.F/PAN**	HRP2 + lactatodehydrogenase (pLDH) P. f. + mix of P.v./P.o./P.m. 15-30 min whole blood (finger-prick)
Optima Palutop+4	HRP2 (P.f.), LDH (P.v.), pLDH P.f. + P.v. + mix of P.o./P.m. whole blood (finger-prick)
This work Multimal	DNA-based P.f + P.v. + P.o. + P.m. + P.k. 40 min **saliva (non-invasive) and trace levels of parasites**

Abbreviations in P. correspond to the different *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*.

*Immuno assay based

4. PROJECT RESULTS

Several results were achieved: 1. Development of a sensor fabrication that is compatible with the upscaling at industrial levels. 2. Test of the sensors with controlled matrices and artificial DNA. 3. Test of the sensors with complex matrix (saliva) and matrices interferences. 4. Extraction of DNA from parasites, which will still be used to test the sensor with saliva and correlation of parasites versus DNA.

4.1. Fabrication results

A fabrication process with 7 lithography steps was developed to ensure the manufacture of the sensors and suitable full-coverage nitride passivation letting open only the graphene sensor. A similar fabrication work was described in previous works [2]; the present work provided improvements on passivation, yield, and uniformity of the sensors properties. The chips were characterized at the wafer level showing that a large majority of the sensors show good electrical characterization properties, as shown on Fig. 1.

4.2. Sensor characterization with spiked buffer and saliva

The sensors were functionalized according to our published procedure [3]. The sensors were calibrated in phosphate buffer (PB) spiked with synthetic DNA in order to evaluate the analytical performance. The sensors

showed consistent responses, starting in the attomolar range. Fig. 2 shows an example of a calibration in buffer. The readings were collected within seconds for all the sensors.

Complex matrices as saliva were tested by performing the experiments using commercial saliva samples spiked with 1 µM of synthetic DNA sequence of *Plasmodium falciparum* fully complementary to sequence immobilized on the graphene surface.

The results show the different saliva tested all show the same tendency, with shifts in signal enabling detection. Results were similar with raw saliva and pre-treated with charcoal.

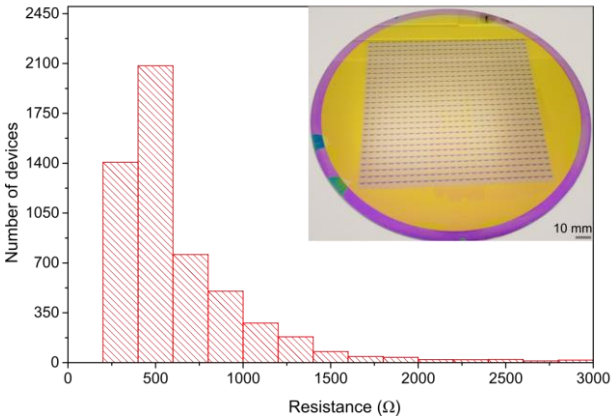

Fig. 1. Electrical characterization of a subset of 8624 sensors from a fabricated wafer. In inset, picture of a 200 mm wafer with 784 sensor chip containing each 20 sensors.

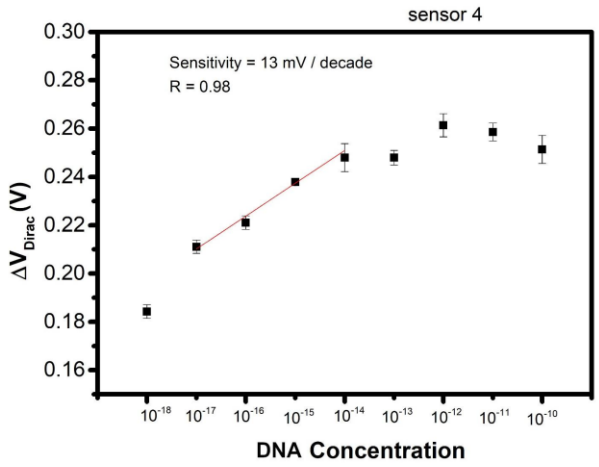

Fig. 2. Calibration curve of the sensors functionalized with probe for *P. falciparum*, tested with matching DNA in buffer, showing a sensitivity of 13 mV/decade and a limit of detection lies the attomolar range.

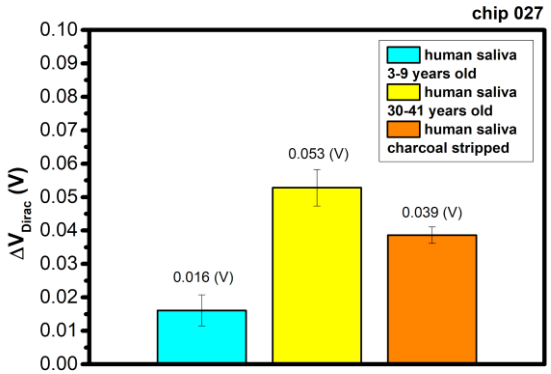

Fig. 3. Sensor response using different commercial saliva samples spiked with 1 µM of target DNA for *Plasmodium falciparum*. All salivas tested yield a shift of the signal enabling a conclusion on a positive test.

4.3. DNA extraction results

The validation study was started by PCR analyses of *Plasmodium* nucleic acids in the pico-attomolar range, to infer the lower limit of *Plasmodium* DNA quantification. For this, DNA from a pooled sample of 256 parasites was evaluated by PCR. Sequential dilutions were performed and *Plasmodium* DNA was detected down to 1 parasite. This result shows that we enable future correlations of the sensor results with PCR-obtained data down to 1 parasite.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

The fabrication of the major part of the silicon disposable consumable is currently performed in wafer of close to 1000 sensor chips with a lead time of 2 to 3 weeks. Improvements of the fabrication process to be implemented in a second phase will enable manufacture of several wafers simultaneously, leading to hundreds of thousands of sensor chips annually. Beyond the production of the silicon part, the fabrication factors represent limits to the throughput are: 1. production of graphene monolayer; a machine upgrade would drastically decrease graphene growth time, multiplying the production; 2. wire-bonding, where the current use of a semi-automated machine limits the throughput to a few thousands annually, requiring investment in an automated system or sub-contracting this step; 3. spotting of DNA targets onto the chip, requiring an upgrade of our machine to increase throughput beyond a few thousand per year.

In terms of the clinical trials, feedback from clinicians (TRL 4 diagnosis device, according to [4]) are being gathered starting in the local hospital of Braga on a few tens of tests, later in hundreds or thousands in international partners.

The protocol for sample pre-process (TRL 5) will continue to be optimised at the University of Minho in contact with foreign partners.

The quality controls (TRL 6-7) will be established once the protocols are complete, and documented in view of future ISO 13485 certification, serving as basis for the regulatory package.

5.2. **Project Synergies and Outreach**

Partners for clinical testing include previously established partnerships, the Clinical Academic Center from the hospital of Braga (Portugal), the National Institute of Research in Tribal Health (India). For the development in software and hardware the Royal Institute of technology of Stockholm (Sweden) is considered. The project team has already worked with these foreign partners on follow-up proposals during the time of this project.

The civil society will be involved including an NGO assisting malaria patients, gathering feedback on user acceptance and data protection concerns. A potential partner is a voluntary work effort between Dundo (Angola) and the University of Minho.

During phase 2, the consortium will reinforce the existing effort for communication developed during phase 1, which included: an interview for national news agency Lusa [5], a television interview [6], and an online event [7].

5.3. Technology application and demonstration cases

The project will benefit EU citizen living in outermost regions of the EU where malaria is endemic [8]: 212 locally-acquired malaria cases were diagnosed in French Guyana in 2019 [9]; citizen in mainland EU regions with vector mosquitoes, such as Italy and France local where cases of Chikungunya and Zika are detected [10]; citizen travelling abroad, bringing 6017 imported malaria cases in 2014 [11].

In phase 2, the project will demonstrate its technology in four steps.

- develop a more integrated electronic reader and associated software, together with partners specialized in usability;
- upscale the production of the consumable in numbers adequate for clinical trials, including automated DNA micro-spotting of the chips, and implement of quality controls;
- set-up a clinical trial, in a hospital in Europe and in a place where malaria is endemic;
- extend the concept to another tropical disease present in malaria endemic region.

As a final product, the demonstrator will include a pocket reader, documentation and consumables for the case of the malaria and another infection active in the selected region. The regulatory package will be prepared.

The project envisions a twofold contribution to the Research infrastructures. The technology was optimized at INL, the only intergovernmental organization dedicated to nanotechnology. The beginning of a production would improve infrastructure use and lead to investment in equipment, benefiting its research capability. As the project intends to gather data regarding the malaria disease in endemic countries, data packages will be submitted to public platforms, such as that of EMBL-EBI.

5.4. Technology commercialization

The team envisions the creation of a start-up to fabricate and distribute the product. The project contracted counselling on intellectual property, and hired services in product design to make a first non-functional mock-up that can be showcased.

5.5. Envisioned risks

A technological risk concerns the low levels of DNA in saliva in sub-clinical malaria. A single-step amplification could be used as a mitigation strategy [12].

A social risk is patient acceptance, due to the different look and feel from existing paper-based tests. Product design can consider similar guides as electronic pregnancy tests to display a more familiar look.

5.6. Liaison with Student Teams and Socio-Economic Study

In phase 2, a member of the team will be in charge of management of the communication with students in management; information exchange will be organized between MSc and science students working on the project and experienced team members, including written documentation and videos. The students will be involved in the market study. During the implementation of a quality management system, students with a suitable profile in quality management will be invited on premises to participate in an on-site internal audit. This will also bring the occasion to involve interested students in the development of documentation for the regulatory approvals.

The team will also contribute to the socio-economic study of the ATTRACT through the communication of the project activities, for enhancing public awareness, by TV, radio, podcasts and newspapers. The social networks will also be used as channels to reach society and contribute with relevant data for the study.

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