Nanodisc-electrode array for single particle detection of tumor derived extracellular vesicles in blood (NanoDisc)

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ABSTRACT

The main aim of NanoDisc project is the label-free electrochemical detection of tumor-derived extracellular vesicles (td-EVs) in blood with unprecedented sensitivity and specificity. The long-term goal is to deliver an analytical platform to monitor treatment efficacy at the point of care diagnosis of cancer. During this project we have optimized the fabrication of nanodisc-electrode array, surface conjugation of the antibody for specific detection and successfully detected single (individual) tumor-derived extracellular vesicles using the electrodes. Detection of tumor-derived extracellular vesicles to such low levels can improve the cancer patient management in the future.

Keywords: tumor-derived extracellular vesicles; label-free electrochemical detection; nanoelectrodes.

1. INTRODUCTION

A challenge in cancer patient care is to monitor the response to treatment.[1] Nowadays, most of the established approaches use tissue staining, which implies their invasive collection through, e.g., biopsies of the primary tumour. Patient follow-up therefore requires serial biopsies, making it very unpleasant for the patient. Alternatively, in vivo imaging approaches using magnetic resonance imaging (MRI) and positron emission tomography–computed tomography (PET-CT) are used. However, the main limitation of imaging techniques is the resolution limit that they can only detect tumours of about 1 cm or bigger. Moreover, high cost, radiation problems (for PET-CT), and/or allergic reactions render these tests unsuitable for repeated use. Nonetheless, more frequent monitoring of the patient to study the treatment efficacy is crucial for making a more accurate prognosis and prescribing personalised treatment. Liquid biopsy is an alternative technique to surgical biopsies and imaging methods which helps to find a range of tumor information through a blood sample. It is non-invasive, rapid, precise, and especially real-time.[2]

Currently, circulating tumor cells (CTC) are being used for liquid biopsies. In this project we examined tumour-derived extracellular vesicles (td-EVs), since they are much more available in blood compared to CTCs ($\backsim 10^3$ higher).[3] EVs are small (50 nm – 1 μm), membrane-enclosed carriers, which are produced by all cells. Nonetheless, the limit of detection (LOD) of td-EVs using state-of-the-art techniques is several orders of magnitude higher than their clinically relevant concentrations (less than 10^4 td-EVs/ml) in blood.[4] Consequently, to have a precise quantification of td-EVs, an ultra-selective device is required, that can also measure ultralow concentrations.

Here, we used an array of nanoscale electrodes for the label-free electrochemical detection of td-EVs with high sensitivity, down to the single td-EV level. One of the main challenges at such low concentrations however, is the diffusion time of the biomarkers to the electrodes, which is relatively large for EVs. Microfluidics can help to reduce this problem to some extent. Nonetheless, a better method is required to attract EVs onto the electrodes. One of the inherent features of these electrodes and the measurement technique is that, the electrophoretic force generated during the oxidation reaction at the nanoelectrode, attracts td-EVs onto the electrode, making the transport faster. Expectedly, other biomolecules will also be attracted to the nanoelectrode. Therefore, an antifouling layer is necessary on the electrodes to ensure highly selective detection. For specific detection, the nanoelectrodes will be functionalised with antibodies targeting td-EVs and that is in progress now. Thus these nanoelectrode sensors can provide a robust and easy-to-use, non-invasive biosensing platform, for both highly sensitive and selective cancer marker detection in blood.

2. STATE OF THE ART

Liquid biopsies are highly promising for metastatic cancer disease management.[2] In this noninvasive approach, a sample of blood (typically a few mL, e.g., from a finger prick) is screened for the presence of tumor biomarkers, such as circulating tumor DNA (ctDNA), miRNAs, tumor-derived extracellular vesicles (tdEVs), or circulating tumor cells (CTCs).[4] The concentration of both CTCs and tdEVs increases with the progression of the tumor.[5] One of the FDA-approved technologies, CellSearch®, for CTC analysis for cancer diagnosis, prognosis, and patient management for certain types of cancer, quantifies CTCs after immunomagnetic enrichment of patient blood samples using fluorescent immunostaining. However, it has been suggested that to confidently conclude that CTCs are absent, 1 litre of blood must be analysed.[6] Occluded by billions of red blood cells and millions of white blood cells, only a few (typically <10) CTCs are present per mL of blood. The scarcity of CTCs hinders the statistical robustness of these measurements. The widespread tdEV concentrations naturally occurring in blood make them promising alternative cancer biomarkers. The variation between patients with low and high tdEV abundance is relatively much higher than for CTCs (a factor of 10^6 for EVs vs. a factor of maximally $\sim 10^3$ for CTCs), and therefore statistically more unlikely to yield false-negative results.[7] If an accurate technique for the quantification of td-EVs in blood would exist, real-time, personalized therapy adjustments could be based on statistically much more reliable liquid biopsy results. To enable accurate quantification of td-EVs from patient blood, an ultrasensitive and ultraselective device is required, to detect individual submicron particles at attomolar concentration (with $\backsim 10^3$ EVs ml^{-1} resolution). Previous attempts for td-EVs detection at low concentrations have reported LOD of 10^5 td-EVs ml^{-1} or more.[4] Conventionally, the EVs are immunocaptured on a surface, and then detected using *e.g.* fluorescence microscopy. Amperometric devices like glucose sensors have been a commercial success for decades. Here, we propose a real-time biosensing method using our NanoDisc technology, based on electrochemical measurements, which are fast, label-free and modelled to sense ultra-low td-EV concentration (down to the single EVs levels) with distinguishable differences between td-EVs and other EVs in blood resulting in less false positives. NanoDisc technology will make a breakthrough by detecting td-EVs at physiologically relevant concentrations for the first time.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

Evaluation of the treatment efficacy for cancer patients and patient follow-up after tumour removal, costs billions of euros each year for different types of cancers. State-of-the-art diagnostic methods include tissue staining involving biopsy. These complex follow-up techniques are quite expensive restricting the number of follow-up tests per year. The current global market for non-invasive cancer diagnostics alone is assessed around $110 billion in 2016. Alternative, non-invasive techniques are still expensive and time consuming. Nonetheless, more frequent (e.g., several times per month rather than several times per year) monitoring is essential for preventing the disease recurrence and progression. This demands non- or minimally invasive techniques that are more accurate, fast and simple. Our NanoDisc technology fulfils all these requirements, and can as such become an innovative and ground-breaking alternative. Our unique feature is the faster monitoring by highly specific single td-EV detection. The direct customers for such a diagnostic device will be general laboratories, clinical pathologists, oncologists, general physicians etc., catering to millions of patients each year.

The value propositions of the technology are sensitive and reliable patient monitoring, with non- (or minimally) invasive sample collection. NanoDisc will also be instrumental for personalised treatment, prognosis, and with population screening for early-stage cancer diagnostics among the high-risk groups. Importantly, this technology is not limited to td-EV detection from blood or other body fluids, it can easily be extended to the detection of DNA, viruses and/or bacteria by simply replacing the recognition elemen (*e.g.* antibodies). In fact, while the focus will initially be on health care applications, NanoDisc technology could equally be used to analyse e.g. oil, sea water, or any liquid in the food or cosmetic industry. Furthermore, we have started three clinical proof-of-concept studies since ATTRACT project began (cancer diagnosis based on td-EV detection, bacterial detection for Chronic obstructive pulmonary disease (COPD) diagnostics, SARS-CoV2 virus detection for COVID-19 diagnostics).

4. PROJECT RESULTS

The key-objectives NanoDisc project were

- Fabrication and electrochemical validation of nanodisc electrodes
- Optimisation of a surface functionalization strategy that combines generic anti-fouling moieties with covalently-bound antibodies
- Development of an experimental protocol, including optimization of the measurement conditions

- Demonstration of the ultra-sensitive td-EV detection in buffer, serum, blood etc. down to a few-td-EV level.

Fabrication and validation

The fabrication of nanodisc electrodes were done using standard clean-room fabrication methods involving micro/nano-lithography, physical vapour deposition, chemical vapour deposition, and etching. Fig. 1 shows the electrochemical validation of the nanodisc electrodes using cyclic voltammogram. It shows the change in the steady state current with respect to the size of nanodisc electrodes.

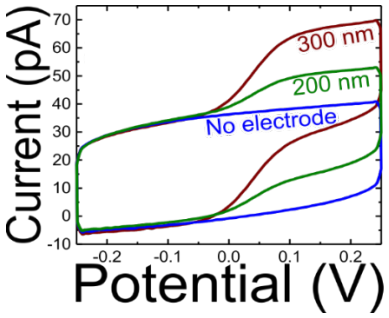

Fig. 1. Cyclic voltammogram (for electrochemical validation) of nanodisc electrodes showing characteristic increase in current to nanoelectrode area.

Surface functionalization

Electrode surface was functionalized with self-assembled monolayers of amine (-NH2) terminated thiol to which a layer of anti-fouling polyethylene glycol chain is attached. Anti-EpCAM antibodies are subsequently conjugated to this layer. In order to optimize the surface functionalization before applying it onto the electrode, we first functionalized flat metal surface using the same method. As shown in AFM images in Fig. 2, tdEVs were successfully and specifically captured on the antibody-conjugated surface. Quasi-spherical objects in the 100 nm – 1 μm size range were identified on the surfaces showing the immobilization of td-EVs . Contrastingly, in negative controls, for which one step of surface functionalization was omitted or which were not exposed to tdEV sample nothing was captured on the surface. After the optimization the same method was used on the electrode for conjugating the antibodies..

An experimental protocol including optimization of the measurement conditions was developed subsequently. Using this protocol together with optimized fabrication technique of the electrodes, we were able to detect individual td-EVs on the electrodes aspecifically. Since the technology is patent pending, these results are not included in this report. The next sept of the project was specific detection of td-EVs in buffer, serum and, blood. However, due to the COVID-19 pandemic, the progress of the project was also affected. Nonetheless, we anticipate that in the coming months, the ultra-sensitive and specific td-EV detection in buffer, serum, blood down to a few-td-EV level could be demonstrated. The details of this method will be published as patent and the experimental results will be published as an article elsewhere.[8]

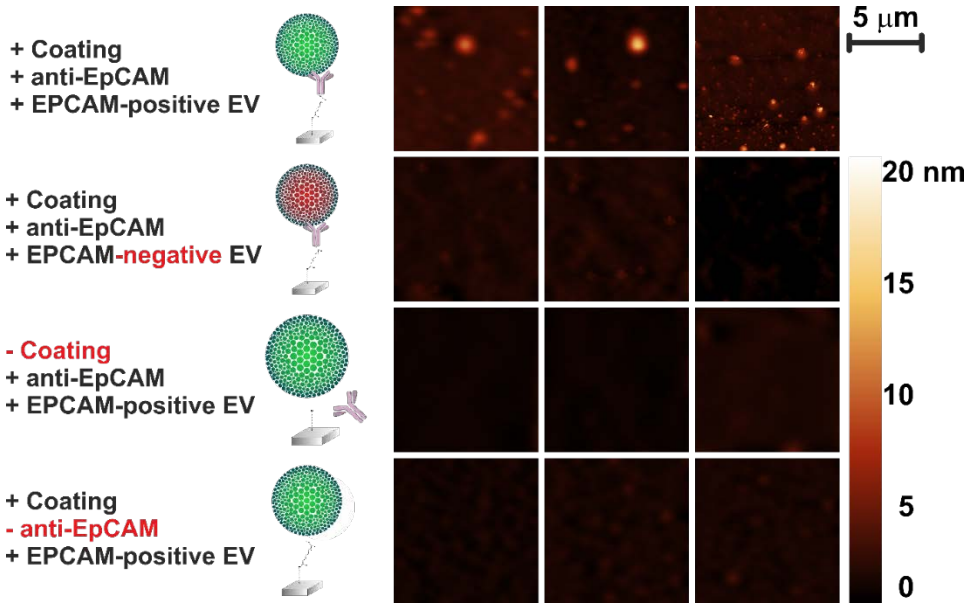

Fig. 2. Atomic force micrograph showing tdEV capture and negative control experiments. The successful capture in the positive control sample compared to the lack of EVs in the negative controls demonstrates the necessity and the performance of each individual surface functionalization step (chemical coating, antibody immobilization) and the performance of the antibody.

1. FUTURE PROJECT VISION

The technology developed in this ATTRACT project has a potential to become a versatile tool for a range of biotechnology applications. In other fields outside of clinical biosensing, there is also a need for highly sensitive electrochemical detection. The straightforward fabrication and operation of our technology make it ideally suited to integrate it in a range of other systems. A follow-up project will aim to push the boundaries of the applicability of Nanodisc technology in order to get it ubiquitously implemented.

1.1. Technology Scaling

Following common TRL definitions, we state that Nanodisc technology is currently at TRL 4; we obtained exciting. We are currently preparing for demonstrating the technology with clinical samples, and negotiations with potential manufacturers have started, in preparation for the development of mass-producible products. Beyond that, in ATTRACT Phase II, we aim to implement the sensing technology in other sensing platforms, to enable more in-depth, fundamental biological research for industrial applications (*e.g.* pharmaceuticals).

1.2. Project Synergies and Outreach

A collaboration is proposed with the team responsible for an ATTRACT Phase 1 project working on single ion channel spectroscopy (Johannes Kepler University, Linz, Austria and CNRS, Paris, France/Tokyo, Japan) as well as with a professor from University of Twente specializing in ion channel studies from a clinical perspective. Together, a system will be developed for in-situ multi-modal analysis of biomolecules. This system can be applied to diagnostics of a range of diseases as well as to drug development and fundamental studies of pathological pathways.

1.3. Technology application and demonstration cases

As a planned outcome of ATTRACT phase I, the first demonstration case will be that of successful clinical trials, where the potential of our technology for cancer diagnostics, COPD and virus testing will be demonstrated. This will happen in conjunction with ATTRACT Phase II, whereby the applicability in other fields will be demonstrated, including scientific research whereby fundamental biological phenomena will be characterized with unprecedented accuracy to help understand physiological processes and to enable development of novel therapeutic strategies.

Contributing both at the fundamental level as well as at the clinical level, the technology developed in ATTRACT Phase I will contribute to the wellbeing of European citizens for decades to come as well as to the efficient organization of health care by developing smart screening tools.

By forming a powerful research consortium with renowned institutes from several member states and including industrial partners with proven track records, a multidisciplinary entity is formed that can function as a vehicle for efficient interfacing with existing Research Infrastructures.

1.4. Technology commercialization

There is a strong commitment among project partners to bring this technology to the clinical market as soon as possible. As much as three clinical proof-of-concept studies have started since ATTRACT Phase I began and discussions with potential suppliers of a mass-producible device are ongoing. A spin-off company, ECsens B.V. has been incorporated to foster these activities. Commercially, the most promising application is for rapid, on-site corona virus testing. Given the impact corona-related restrictions have had on the European economy, partial solutions to newly arisen problems, including widespread testing, have seemingly limitless potential for commercial success. Following recent media coverage about Nanodisc technology, there has been a wealth of interest both from venture capitalists as well as informal investors (business angels).

1.5. Envisioned risks

Societal embedding of the technology is a major concern. To efficiently function as a rapid test, it would be ideal if test results could be linked to personal information and included in local or central databases. This entails significant privacy ramifications. From a clinical perspective, interpretation of test results is an issue: large datasets with in-depth results from many patients should be studied before it is possible to confidently draw any reliable conclusions from data generated by Nanodisc technology. Trust from the clinical community is an important parameter for acceptance of these biosensor devices by health care professionals. Their willingness to adapt to new methods will determine the widespread application of this novel diagnostic approach.

1.6. Liaison with Student Teams and Socio-Economic Study

Students are a valuable source of inspiration for any innovative undertaking. Throughout this ATTRACT project, project partners have been very open to input from individual students in courses, graduation assignments and student teams. The project leader from the following ATTRACT Phase II project will likewise

be enthusiastic towards student team projects organized by the programme. Time invested in such projects is typically earned back quickly.

As explained in the previous section, socio-economic phenomena will play a deciding role in the success of such a promising technology. As such, expert-driven studies are warmly welcomed. Besides contributing to e.g. interviews and collaborating with these studies, project partners from the ensuing ATTRACT Phase II project can even aid in the organization of such studies.

2. ACKNOWLEDGEMENT

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