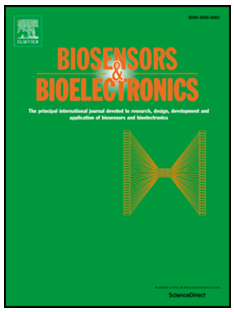

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Hazal Kutluk, Richard Bruch, Gerald Urban, Can Dincer

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IMPACT OF ASSAY FORMAT on miRNA SENSING: ELECTROCHEMICAL MICROFLUIDIC BIOSENSOR for miRNA-197 DETECTION

Hazal KUTLUK[1], Richard BRUCH[1,2], Gerald URBAN[1,3], Can DINCER[1,2]

[1]University of Freiburg, Department of Microsystems Engineering, GERMANY,
[2]Freiburg Center for Interactive Materials and Bioinspired Technologies, GERMANY and
[3]Freiburg Materials Research Center, GERMANY

Corresponding author: Can Dincer, **E-mail:** dincer@imtek.de, **Address:** University of Freiburg, Freiburg Center for Interactive Materials and Bioinspired Technologies, Georges-Köhler-Allee 105, Room 02-028, 79110 Freiburg, Germany

AUTHOR CONTRIBUTIONS

Hazal Kutluk: Methodology, Conceptualization, Validation, Formal Analysis, Investigation, Resources, Writing – Original Draft, Visualization. **Richard Bruch:** Methodology, Resources, Writing – Review & Editing, Supervision. **Gerald Urban:** Funding Acquisition, Supervision. **Can Dincer:** Conceptualization, Methodology, Resources, Writing – Review and Editing, Supervision, Project Administration, Funding Acquisition.

ABSTRACT

MicroRNAs (miRNAs) are important biomarkers for the early detection of various diseases, especially cancer. Therefore, there is a continuing interest in different biosensing strategies that allow for the point-of-care measurement of miRNAs. Almost all miRNA sensors utilize cross-hybridization of the target miRNA with a capture probe for the recognition, which can be designed in either a sandwich or a competitive format. In this work, we present a low-cost microfluidic biosensor platform for the electrochemical measurement of miRNA-197 (a tumor biomarker candidate) in undiluted human serum samples, using very low sample volumes (580 nl) and within an hour. For this purpose, different on-chip miRNA bioassays based on sandwich and competitive formats are developed and compared in terms of their sensitivity, dynamic range, selectivity, precision, and simplicity. The obtained results show that, despite having a narrower dynamic range when compared to the competitive format, the sandwich assay has superior performance regarding its sensitivity and selectivity. The lowest limit of detection which can be achieved with the sandwich assay is 1.28 nM (0.74 fmole), while 4.05 nM (2.35 fmole) with the competitive format. Moreover, the sandwich assay proves to have a better distinction against single-base mismatch oligonucleotide sequences compared to the competitive one. Due to its versatility and easy handling, overcoming the issue with the sensitivity, the implemented electrochemical microfluidic biosensor could pave the way for rapid and low-cost on-site miRNA diagnostics.

KEYWORDS

microRNA analysis, competitive assay, sandwich assay, electrochemical biosensors, microfluidics, point-of-care testing

1. INTRODUCTION

Almost three decades after their discovery, microRNAs (miRNAs), 18-25 nucleotide long, non-coding RNA sequences, are known to be a crucial part of the post-transcriptional gene regulation in eukaryotic cells, estimated to be regulating 30% to 50% of all human genes (Mack, 2007; J. Wang et al., 2016). Dysregulation of miRNAs in the human body is related to many diseases, such as cardiovascular, neurodevelopmental, autoimmune disorders and cancer (Ardekani and Naeini, 2010; D. Wang et al., 2016). Moreover, miRNAs are declared as possible drug agents, drug targets, and importantly, biomarkers for the early detection of diseases (Anthiya et al., 2018; Rupaimoole and Slack, 2017; Schmidt, 2014; J. Wang et al., 2016). All these aspects result in a great interest in miRNAs and the potential ways to detect them in blood, serum or cell samples in the most sensitive and selective way possible, leading to the development of a wide variety of detection strategies.

Among all reported detection methods, systems enabling point-of-care testing (POCT) have gained increasing significance in the scientific world. POCT is defined as “diagnostic testing at or near the site of the patient care, wherever that medical care is needed” (Kost, 1995). Contrary to conventional miRNA detection methods, such as northern blotting, quantitative reverse transcription polymerase chain reaction (RT-qPCR) or microarrays, POCT devices do not require heavy equipment, a centralized laboratory environment, or highly educated personnel to carry out the procedure (Hunt et al., 2015; Shaw, 2016). Therefore, although there are still practical challenges, POCT presents an important benefit to clinical diagnostics, since it can be used in resource-limited settings, like in developing countries, in doctors’ offices or directly at home (Dincer et al., 2017; Lopez-Barbosa et al., 2016; Shaw, 2016; Silveira et al., 2016; S. Wang et al., 2016). Through on-site testing, medical services can be made available to a greater population and early diagnosis of diseases can be promoted. Moreover, POCT can help reduce the costs of testing and minimize the need for specialized equipment as well as reducing the sample volumes.

A wide range of transduction and detection mechanisms can be employed in biosensing systems, such as optical, electrochemical, thermometric, piezoelectric, magnetic or calorimetric (Dincer et al., 2019; Keshavarz et al., 2015). Electrochemical biosensor approaches, due to their simplicity and versatility, as well as their potential in delivering low-cost, rapid, sensitive, selective and multiplexed measurements, are widely preferred for POCT approaches, as well as the detection of miRNAs (da Silva et al., 2017; Dincer et al., 2017; Hamidi-Asl et al., 2013; Keshavarz et al., 2015; Labib and Berezovski, 2015; Ozsoz, 2012; Wang, 2006). Moreover, electrochemical biosensors can be miniaturized, which potentially makes them an even more appropriate choice for personalized medicine and point-of-care applications (Ozsoz, 2012; Teo et al., 2014).

In general, an electrochemical miRNA biosensor comprises of two main elements, namely, an element responsible for the biological recognition of the miRNA, and a transducer element generating a measurable signal as a result of this bio-recognition (Kilic et al., 2018). Almost all biological miRNA detection approaches are based on the hybridization of the target miRNA with a capture probe, where the capture probe is a nucleic acid of complementary nucleotide sequence (Cissell and Deo, 2009; Johnson and Mutharasan, 2014). Here, though, the designed capturing format of the implemented biological assay might play a significant role in the biosensor performance. One simple way to capture the target miRNA, for instance, is to allow for direct hybridization with the target's complementary sequence. In this format, the target miRNA molecules might need to be labeled with an additional molecule, such as biotin, to enable signal generation (Kilic et al., 2012). If the capture configuration is based on the competition of the unlabeled miRNA with a labeled tracer for a limited number of binding sites, the format is referred to as being "competitive" (Diamandis and Christopoulos, 1996; Sittampalam et al., 2004). Contrarily, the analyte can be "sandwiched" between a layer of capturing probes and a layer of detection probes, which can bind to the analyte at different binding sites. This non-competitive format is mostly referred to as the "sandwich" format (Diamandis and Christopoulos, 1996).

It is of importance to note that, traditionally, the competitive and sandwich assay formats are realized by producing analyte-specific antibodies and making use of the high affinity and specificity between these two elements (Diamandis and Christopoulos, 1996; Van Emon, 2007). The theory and performance of the competitive and sandwich assay configurations employing this antibody-antigen binding have been widely studied, and possible potentials and drawbacks of each assay structure are commonly evaluated (Diamandis and Christopoulos, 1996; Ecker et al., 2013; Jackson and Ekins, 1986; Omi et al., 2015; Price and Newman, 1991; Prudom et al., 2010; Tijssen, 1985; Wild, 2013). Sandwich assay, for instance, is shown to be more sensitive than the competitive assay in theory and practice, and it is as well expected to be more specific than the competitive counterpart, due to the use of two distinct antibodies to capture the analyte (Ecker et al., 2013; Jackson and Ekins, 1986; Omi et al., 2015; Prudom et al., 2010; Tijssen, 1985). On the other hand, a sandwich assay cannot be used to detect small molecules, which, structurally, do not have two distinct epitopes which allow for the binding of two different antibodies, and therefore, a competitive assay is needed (Omi et al., 2015).

The detection strategy of miRNAs, though, as mentioned before, are based on the hybridization of the miRNAs with a complementary single-stranded DNA (ssDNA) or an RNA sequence, instead of employing antibodies against the miRNA of concern. This raises the question of whether the sandwich or the competitive assay shows superior performance in miRNA detection. Although there have been a few studies evaluating the performance of the competitive and sandwich assays through the assessment of kinetics and/or thermodynamics of nucleotide hybridization processes, to the best of our knowledge, there is no study that compares these two methodologies experimentally on the same system (Cherepinsky et al., 2010; Miranda-Castro et al., 2018). Therefore, in this work, our purpose is to translate the knowledge generated on sandwich and competitive assay formats using antibody-antigen interactions, and directly compare the performances of sandwich and competitive assays on the same system in terms of selectivity, sensitivity, dynamic range, reproducibility, and handling.

Some recently presented electrochemical biosensors employing competitive and sandwich assay formats and enzyme-based signal generation for the detection of miRNAs are given in table 1, as well as the details of the current work for comparison purposes. Here, it is seen that the presented systems use mainly horseradish peroxidase (HRP) and alkaline phosphatase (AP) as enzymes and different electrochemical detection strategies, such as amperometry and differential pulse voltammetry. The electrode surface is increased in all studies through nanosheets and/or nanoparticles to allow for a more sensitive measurement. In competitive miRNA assays, as a capture probe, both RNA and DNA sequences are used, whereas in sandwich format, DNA capture and detection probes seem to be the dominant choice. The required sample volumes for the proposed biosensors change in a range between 8-100 µl and the limits of detection between 0.05 fM-0.2 nM.

In this work, we utilize a low-cost, disposable and highly versatile microfluidic biosensor platform for the electrochemical detection of miRNA-197 in undiluted human serum samples. The proposed microfluidic biosensor platform, in contrast to the presented state-of-the-art, does not require an extensive electrode modification using different micro/nanomaterials, which makes its production easier, cheaper and more suitable for high-throughput applications. Moreover, it requires only a sample/reagent volume of 580 nl and offers the possibility for a multiplexed detection of various miRNAs simultaneously (Kling et al., 2016).

As the target miRNA, whose concentration is of interest, the 22-nucleotide long miRNA-197 is chosen, which is being studied on a biosensor platform for the first time. Dysregulation of the miRNA-197 is believed to play an essential part in various cancer types such as pancreatic, lung or colorectal cancer, and therefore, it is described as a novel biomarker for cancers (Cheng, 2015; Shaker et al., 2015; D. Wang et al., 2016).

Table 1: *Overview of some of the recently presented miRNA detection methods based on competitive and sandwich assays*

Work	Signal detection	Target hybridization	Signal amplification techniques	Electrochemical measurement	Target miRNAs	LOD	Sample volume (µl)	Selectivity	Biological sample
Zouari et al., 2017	Enzyme-based (HRP)	Competitive RNA-RNA hybridization	AuNPs captured on SPCEs	Amperometry	miRNA-21	25 fM	10	Single-base central MM	Total RNA extracted from cells
Vargas et al., 2018	Enzyme-based (HRP)	Competitive DNA-RNA hybridization	Magnetic beads captured on SPCEs	Amperometry	miRNA-21	0.2 nM	25	Single-base central MM, average discrimination against single-base terminal MM	Total RNA extracted from cells
Chen et al., 2017	Enzyme-based (HRP)	Competitive RNA-RNA hybridization	WSe_2 nanosheet modified electrode	Differential pulse voltammetry	miRNA-21	0.06 fM	8	Single-base central MM	Serum samples
This work	Enzyme-based (GOx)	Competitive DNA-RNA hybridization	Stop-flow	Amperometry	miRNA-197	4.05 nM	0.58	Non-complementary sequence	Spiked serum
Mohammadi and Amine, 2018	Enzyme-based (AP)	Sandwich hybridization, anti-miR capture and detection probes	Magnetic beads captured on SPCEs	Differential pulse voltammetry	miRNA-155	29 pM	50	Non-complementary sequence	Spiked serum
Mandli et al., 2017	Enzyme-based (AP)	Sandwich hybridization, DNA capture and detection probes	AuNP modified pencil graphite electrode	Differential pulse voltammetry	miRNA-21	100 pM	100	Non-complementary sequence	-
Shuai et al., 2017	Enzyme-based (AP)	Sandwich hybridization, DNA capture and detection probes	MgO nanoflower and graphene oxide-AuNPs	Electrochemical-chemical-chemical detection	miRNA-21	0.05 fM	8	Single-base central MM	Spiked serum
This work	Enzyme-based (GOx)	Sandwich hybridization, DNA capture and detection probes	Stop-flow	Amperometry	miRNA-197	1.28 nM	0.58	Single-base central MM, average discrimination against single-base terminal MM	Spiked serum

HRP: horseradish peroxidase, GOx: glucose oxidase, AP: alkaline phosphatase, AuNP: gold nanoparticles, SPCE: screen-printed carbon electrode, WSe_2: tungsten diselenide, MgO: magnesium oxide, MM: mismatch

2. EXPERIMENTAL

2.1. Working principle of the competitive enzyme-linked miRNA assay

Competitive bioassays, as aforementioned, are based on the competition of an unlabeled analyte with a labeled tracer for a limited number of binding places. In this work, the unlabeled analyte, the labeled tracer and the binding places are the target miRNA-197, the fluorescein labeled competitor miRNA, and the immobilized capture DNA probes, respectively. Here, the concentration of the target miRNA-197 is of interest, which is chemically unaltered and in its native form, as it would be found in patient samples.

To implement the competitive assay to the microfluidic biosensor, the microchannel surface is initially functionalized with streptavidin and blocked with bovine serum albumin (BSA). These are immobilized at the channel surface via passive adsorption. Afterwards, the capture DNA probe, a complementary sequence to the miRNA-197, is introduced to the microchannel. Capture probes are immobilized at the microchannel surface using the streptavidin-biotin interaction, as the 3'-end of the capture probe is functionalized with biotin. To prevent any unspecific binding to any streptavidin which is not saturated with the capture probes, a secondary biotin-blocking step is followed.

When an unknown quantity of target miRNA-197 is mixed with a fixed amount of competitive miRNA-197 and introduced to the channel, presenting only a limited number of capture DNA probes, the amount of bound competitive miRNA-197 is a function of the total concentration of the target and competitive miRNAs according to law of mass action (Hulme and Trevethick, 2010; Kenakin, 2016). Therefore, the higher the amount of the target miRNA-197 in the mixture, the less of the competitor miRNA-197 can bind to the capture probes and the other way around. In a competitive assay, the signal is obtained from the signaling molecule, here, the antibody-glucose oxidase complex (Ab-GOx). Ab-GOx is immobilized onto the competitor miRNA-197 as a result of the binding of the fluorescein label and the anti-fluorescein antibody. When glucose is provided to the channel, the amount of immobilized GOx determines the extent of glucose catalyzed into H_2O_2, as illustrated in figure 1a. In summary, in a competitive assay, the obtained signal is inversely proportional to the analyte concentration, and consequently, the standard curve of a competitive binding assay has a negative slope.

2.2. Working principle of the sandwich enzyme-linked miRNA assay

In the sandwich format of the miRNA assay, the microchannel surface is similarly functionalized with streptavidin and blocked with BSA. The channel surface is then saturated with the primary capture DNA probe, which is biotinylated at the 3'-end, composed of only 11 nucleotides and designed to partially hybridize to the miRNA-197 from 5'-end. After the introduction of the sample, which includes varying concentrations of the target miRNA-197, the channel is saturated with the secondary detection DNA probe, which hybridizes with the remaining nucleotides of the immobilized target miRNA-197. Incorporating a fluorescein label at its 5'-end, the bound detection probe binds the Ab-GOx complex and when glucose is provided to the channel, the amount of immobilized GOx determines the extent of glucose catalyzed into H_2O_2, as shown in figure 1b. Therefore, in the sandwich format, the obtained signal is proportional to the analyte concentration, and the standard curve of the sandwich assay has a positive slope.

2.3. Electrochemical microfluidic biosensor

The electrochemical microfluidic biosensor employed in this work, as depicted in figure 1c, is composed of two main sensor components: (i) an immobilization area, into which the biological assay reagents are introduced, and (ii) an electrochemical measurement cell, which is separated from the immobilization area by a physical hydrophobic barrier. This efficient separation between these two elements allows for the flexible design of a bioassay.

The immobilization area is a microchannel of 580 nl volume, whose filling is realized by capillary forces. To introduce the biomolecules, a 2 µl solution droplet is placed on the inlet opening of the channel. Movement of the droplet stops once it reaches the hydrophobic barrier, and, in this way, the flow of the liquid over the electrodes, hence, contamination of the electrochemical cell, is prevented. For the inhibition of a breakthrough of the stopping barrier, its stopping effect is supported by placing a 2 µl droplet of RNase-free phosphate buffered saline (PBS) on the outlet of the channel. By this, the air is trapped in the microfluidic channel and the forward movement of the liquid is prevented. After each incubation step, the reagents are sucked out through the channel inlet, and a washing step with the wash buffer is performed to remove the unbound biomolecules.

The electrochemical cell comprises three on-chip electrodes, namely the working, reference and counter electrodes (WE, RE and CE, respectively). WE and CE are of platinum, whereas RE is of silver/silver chloride (Ag/AgCl).

The biosensor chips are produced on wafer-level by stacking multiple, differently exposed dry film photoresist layers onto a platinum patterned polyimide layer. Readers who are interested in the production method in detail are directed to the video article of Bruch et al. (Bruch et al., 2017). Produced biosensor chips with pre-immobilized nucleic acid assay can be stored in room temperature up to 3 months, or at 4°C for up to 9 months (Bruch et al., 2019). This a remarkable feature of the proposed biosensor system, when compared to many other electrochemical systems, which show a stability of maximum 3 months (Mohammadi et al., 2019).

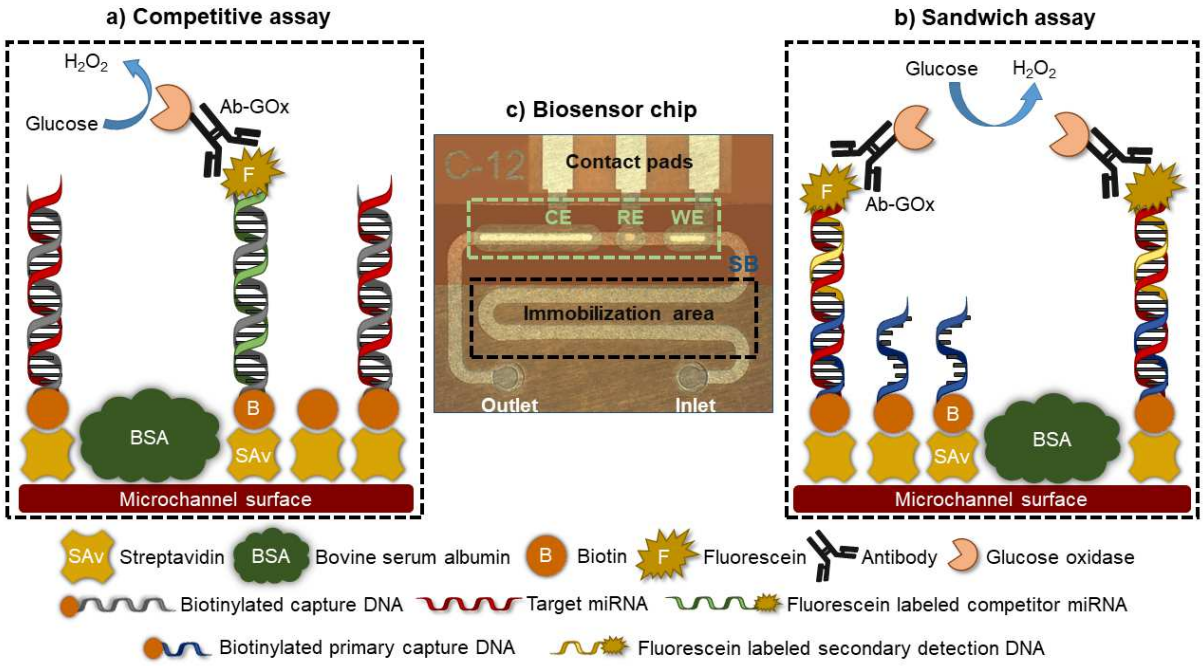

***Figure 1**: Illustration of the **(a)** competitive and **(b)** sandwich assay formats that are employed for the detection of the target miRNA-197 on the microfluidic biosensor. **(c)** Image of the microfluidic biosensor, visualizing the immobilization area (black), the electrochemical cell with the counter, reference and working electrodes (green), and the stopping barrier (SB), shown in blue, which seperates the two chambers.*

2.4. Assay Reagents

The following assay reagents are used for the realization of the competitive and sandwich enzyme-linked assay: RNase-free PBS, wash buffer, streptavidin, BSA, DNA and RNA oligonucleotides, biotin and anti-fluorescein antibodies conjugated with the enzyme GOx.

RNase-free 10x PBS, purchased from ThermoFischer Scientific (USA), is diluted with UltraPure™ DNase/RNase-free distilled water (DI) from the same vendor to achieve a salt concentration of 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4 with a pH of 7.4. Wash buffer, which is purchased from Merck (Germany) in a tablet form, is prepared with UltraPure™ DNase/RNase-free DI to yield 10 mM phosphate buffer, 2.7 mM KCl, 14 mM NaCl and 0.05% Tween at pH 7.4. 1% BSA is used as a surface blocking agent and is purchased from ThermoFischer Scientific (USA).

Streptavidin and biotin are obtained from Merck (Germany) in a lyophilized form and diluted in RNase-free PBS to achieve desired concentrations. Monoclonal anti-fluorescein IgG antibodies, that are also purchased from Merck (Germany), are conjugated with the GOx enzyme with the use of proper conjugation kit (item number: ab102887), which is obtained by Abcam (UK).

DNA and RNA oligonucleotides are acquired from Biomers GmbH (Germany) in lyophilized form and dissolved in RNase-free PBS to produce desired concentrations. Sequences of the used oligonucleotides are presented in Supplementary Information.

2.5. Measurements in human serum

For the spiked serum measurements, a healthy human serum sample is obtained from the University Hospital of Freiburg. To prevent the degradation of the spiked miRNA due to the presence of RNase, 4 U μl^{-1} of murine RNase-inhibitor (purchased from New England Biolabs) is added to the serum (see Supplementary Information). Different concentrations of miRNA-197 are then spiked in this sample and measured with the proposed biosensor platform. The serum sample is used in an almost undiluted form (4:5).

2.6. On-chip immobilization of the competitive enzyme-linked assay

For the realization of the competitive enzyme-linked assay, the microchannel surface is initially functionalized with 1 mg ml^{-1} of streptavidin for 1 hour, then blocked by 1% BSA for another hour. The procedure is then followed by 15-minute incubation steps of the capture DNA probe, 400 µg ml^{-1} of biotin, a mixture of target miRNA-197 and competitor miRNA-197 (sample with synthetic oligonucleotides), and, 8.33 µg ml^{-1} of Ab-GOx. Between each incubation step and at the end of the immobilization procedure, a washing step with 50 µl of wash buffer is carried out and the chips are dried 30 seconds under vacuum.

In order to create a competitive advantage, the target miRNA-197 is introduced to the immobilization channel in a separate incubation step before its competitive analogue. In this case, the biotin blocking step is followed by 15-minute incubation steps of the target miRNA-197, competitor miRNA-197, and Ab-GOx.

For any competitive enzyme-linked assay, the concentrations of the capture DNA probe and the competitor miRNA-197 are kept equal. In this work, the performance of three competitive enzyme-linked assays is evaluated using 100, 50 and 10 nM concentrations for the capture DNA probe and the competitor miRNA-197.

2.7. On-chip Immobilization of the sandwich enzyme-linked assay

For the realization of the sandwich enzyme-linked assay, the microchannel surface is functionalized with 1 mg ml^{-1} of streptavidin for 1 hour, then blocked by 1% BSA for another hour. This is then followed by 15-minute incubation steps of 2 µM of primary capture DNA probe, target miRNA-197 (sample with synthetic oligonucleotides or spiked serum samples), 2 µM of secondary detection DNA probe, and, 8.33 µg ml^{-1} of Ab-GOx. Between each incubation step and at the end of the immobilization procedure, a washing and drying step is carried out, as for the competitive enzyme-linked assay incubation.

2.8. Electrochemical signal readout

The electrochemical signal readout is performed following the on-chip immobilization. The measurement setup used in this work for this signal readout process includes a syringe pump PHD2000 which is obtained by Harvard Apparatus (USA), a four channel potensiostat MultiEmStat3 by PalmSens (The Netherlands), a personal computer with MultiTrace Software, a

custom-made printed circuit board (PCB) as an electrical interface and a custom-made chip holder with silicone tubing, which allows for the microfluidic connection.

Prior to the measurement process, a pre-conditioning of the working electrodes is carried out in order to obtain a residue-free platinum working electrode surface. To achieve that, the electrode potential is cycled between +0.8 and -0.05 V, 5 seconds each, for 30 cycles (see Supplementary Information). During the pre-conditioning, RNase-free PBS is passed through the microchannel at a flow rate of 20 µl min^{-1}.

The amperometric measurement, which is carried out at 0.45 V *versus* the on-chip Ag/AgCl reference electrode, is based on the stop-flow method. Here, the fluid flow (20 µl min^{-1}) in the microchannel is stopped for a defined period, hence, the electrochemically active species accumulate in the immobilization area. Upon the restart of the fluid flow, the accumulated species pass over the working electrode, resulting an amperometric signal, which is observed as a peak (Dincer et al., 2016). The fluid used in the measurement process is a 40-mM glucose (Merck, Germany) solution prepared with RNase-free PBS.

2.9. Fitting of assay data and calculation of assay performance parameters

Five-parameter logistic (5PL) curve model is used in this work to fit the assay data. The assay performance is evaluated in terms of precision (represented as the inter-assay coefficient of variation – CV), sensitivity (i.e. LOD), dynamic range (range between lower and upper limits of detection – LOD - ULOD), selectivity, and handling. Details can be found in Supplementary Information.

3. RESULTS and DISCUSSION

3.1. Sensitivity and dynamic range of the competitive assay

The balance between the capture DNA probes and the competitor miRNA plays an essential role in the sensitivity of the competitive assay. In an ideal case, the amount of the capture DNA probes in the microchannel should be equal to the amount of the competitor RNA. If the capture DNA probes are in abundance with respect to the competitor RNA, the target miRNA can bind to these “excess” binding spots without causing a decline in the obtained signal response (see Supplementary Information). On the other hand, if the competitor RNA is higher in amount, there is the possibility that the competition between the competitor and the target probes are negatively affected due to the plentitude of the competitor miRNA. This effect might be enhanced in a competitive assay where the capture probes have similar binding affinity to the target and the competitor molecule.

The sensitivity of the competitor assay is investigated by introducing equal concentrations of the capture DNA probe and the competitor miRNA to the microchannel. The effect of the chosen concentration is demonstrated by using 100, 50 and 10 nM of both components. To obtain a standard curve, solutions containing varying concentrations of the target miRNA-197 and a fixed concentration of the competitor miRNA-197 are prepared and introduced to the biosensor, then the respective signal response of the assay is investigated. The data, as presented in figure 2, is obtained with a 5-min stop-flow protocol, and fitted with a 5-parameter logistic fit.

The calculated LOD and ULOD for each calibration curve obtained in this work are given in table 2. For concentrations of 100, 50 and 10 nM of the capture DNA-competitor miRNA pair (CCP), the calculated LOD is 8.17, 5.89 and 4.05 nM (4.74, 3.42 and 2.35 fmole), respectively. This suggests that there is a slight improvement in the assay sensitivity with the use of lower concentrations of the CCP. On the other hand, with the reduction of the used concentration, the signal response of the assay decreases, which implies that it would not be possible to lessen the used concentration constantly to achieve a higher sensitivity, as the signal would vanish.

The dynamic range indicates the concentration range of the target miRNA, where the response signal can statistically be distinguished from the highest and the lowest signal response, and is defined in this work as the domain between the LOD and ULOD. From table 2, it is seen that the maximum dynamic range is achieved by using 100 nM concentration for the CCP, which covers a dynamic range of roughly three orders of magnitude. For 50 nM and 10 nM CCP, the dynamic range covers two and one orders of magnitude, respectively. This shows that a decrease in the concentration of the CCP leads to a significant narrowing in the dynamic range of the competitive assay, while not leading to a considerable improvement in the sensitivity.

By introducing the target miRNA into the immobilization channel prior to the competitor RNA, an advantage is provided for the target miRNA. The effect of this competitive advantage on sensitivity is investigated by using 50 nM for the CCP. Comparing the current densities of the individual measurement points in the calibration curves provided in figure 2c and 2d, it can be seen that introducing the target miRNA to the microchannel prior to the competitor miRNA causes only a slight decrease in the signal. This is supported by the calculated LOD of both curves, where the assay with the competitive advantage provides an LOD of 4.51 nM (2.62 fmole) compared to 5.89 nM (3.42 fmole) of the prior. This result implies that the capture probes favor neither the target miRNA nor the competitor miRNA for binding, and therefore, a significant improvement in the assay sensitivity cannot be achieved by providing competitive advantage for the target miRNA.

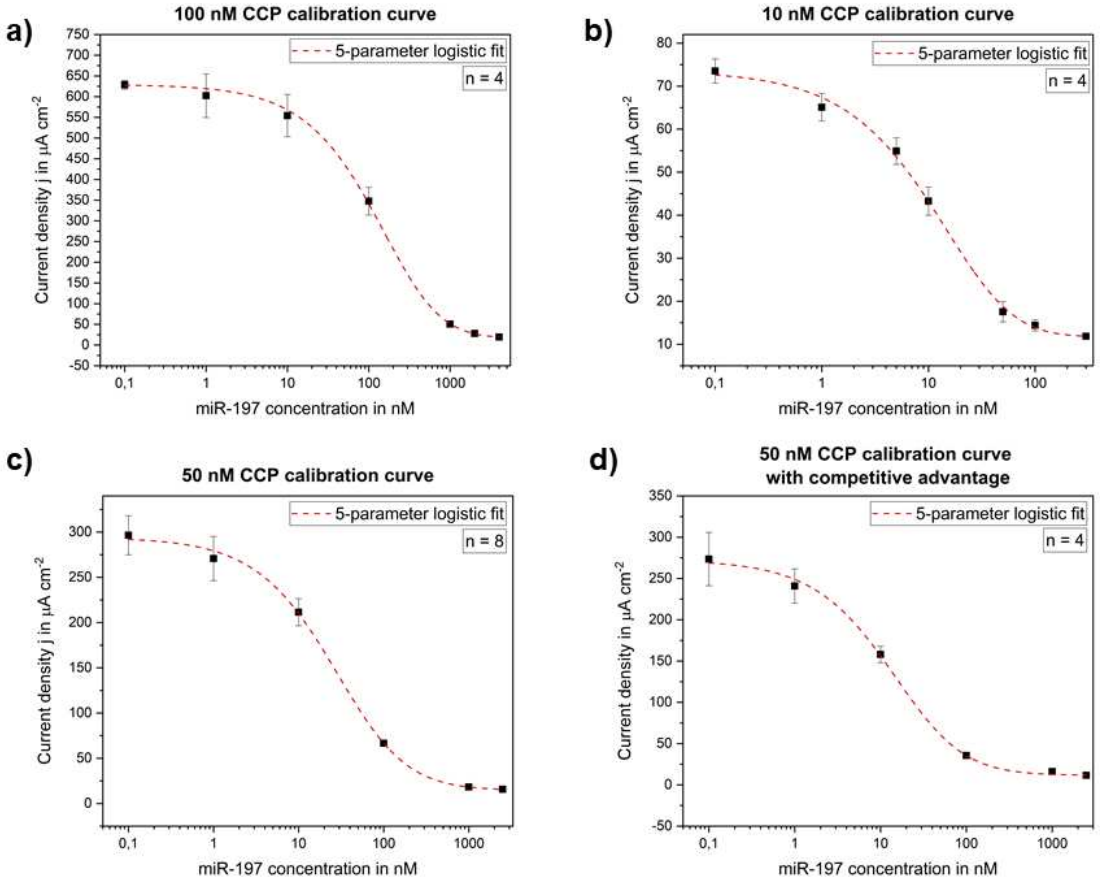

Figure 2: *Competitive assay standard curves with* ***(a)*** *100 nM* ***(b)*** *10 nM* ***(c)*** *50 nM capture DNA-competitor miRNA pair. In* ***(d)****, the standard curve is obtained by providing competitive advantage to the target miRNA. All data is obtained with a 5-min stop-flow protocol and fitted with 5-parameter logistic fit. Error bars indicate ± SD, while the inter-assay CV for all measurement points is below 15%.*

3.2. Sensitivity and dynamic range of the sandwich assay

Sensitivity of the sandwich assay is investigated by examining the obtained signal responses of the assay when different concentrations of target miRNA-197 are introduced. The microchannel surface is saturated with primary capture and secondary detection probes to be able to capture the smallest amounts of the target miRNA-197. Data is obtained with a 5-min stop-flow protocol and is fitted with a 5-parameter logistic fit to consider probable curve asymmetry, as given in figure 3a. An LOD of 1.28 nM (0.74 fmole) is achieved and a dynamic range of one order of magnitude is observed.

To evaluate the accuracy of the calibration curve, spike and recovery method is used for three different target RNA concentrations, namely for 5, 20 and 70 nM. These three concentrations are chosen as a representative of three different regions of the calibration curve: 70 nM for the region of the curve showing a linear change with respect to the target miRNA concentration, 20 nM for the region of the curve where this linear behavior is slowly diminishing and 5 nM for

the vicinity of the detection limit. In figure 3b, the blue columns represent the current signals obtained from the measurement of the spiked samples and the red columns represent the values calculated from the calibration curve given in figure 3a. For 5, 20 and 70 nM, recovery yields of 85%, 96.6% and 101% were achieved. This is a positive indication of the accuracy of the obtained calibration curve for the sandwich assay.

One possible way to increase the sensitivity of the sandwich assay might be increasing the sample volume introduced to the microchannel, while keeping the amount of immobilized capture DNA probes fixed. Increasing the sample volume in this manner leads to an increase in the amount of target miRNA molecules entering the microchannel, hence, for a given target miRNA concentration, an enhancement in the signal response is expected. In the context of the proposed microfluidic platform, such an enhancement in the sample volume could be realized, for instance, by carrying out multiple incubations of the sample containing a fixed concentration of the target miRNA.

To demonstrate this, a target miRNA-197 solution of 10 nM is incubated in the microchannel 4-times successively, without any washing and drying steps in between. The results, which are obtained by a 5-min stop-flow protocol, are presented in figure 3c. With a 4-times successive incubation, it is seen that the average current signals increase from 41.3 to 178.2 μA cm^{-2}. This result is in accordance with the standard curve obtained for the sandwich assay, where the signal response for 40 nM concentration is calculated to be 193 μA cm^{-2}. That means, incubating a sample of 10 nM concentration for 4 successive times behaves almost similarly to incubating a 40 nM sample. Therefore, an increase in the sample volume might lead to enhanced current densities for lower target concentrations, providing an improvement in the assay sensitivity.

On the other hand, practically, carrying out multiple incubations on a regular basis in order to increase the sample volume may not be preferred. This is due to the difficulties the procedure induces, such as the necessity of many incubation steps and the increase in the total assay time. In this regard, for future applications, designing the microchannel dimensions to allow for more sample volume, such as increasing the height of the channel by stacking more DFR layers, or a dynamic incubation using a microfluidic setup should be considered.

In order to prove the clinical validity of the proposed biosensor, murine RNAse-inhibitor is added to undiluted human serum obtained from a healthy patient and the sample is spiked with varying concentrations of the target miRNA. The results are seen to be in agreement with the results acquired in PBS, as shown in figure 4d. Thereby, it is possible to say, that the proposed biosensor can be used in a POCT setting without substantial treatment of the serum or the extraction of the miRNAs, when the sensitivity of the assay is further improved.

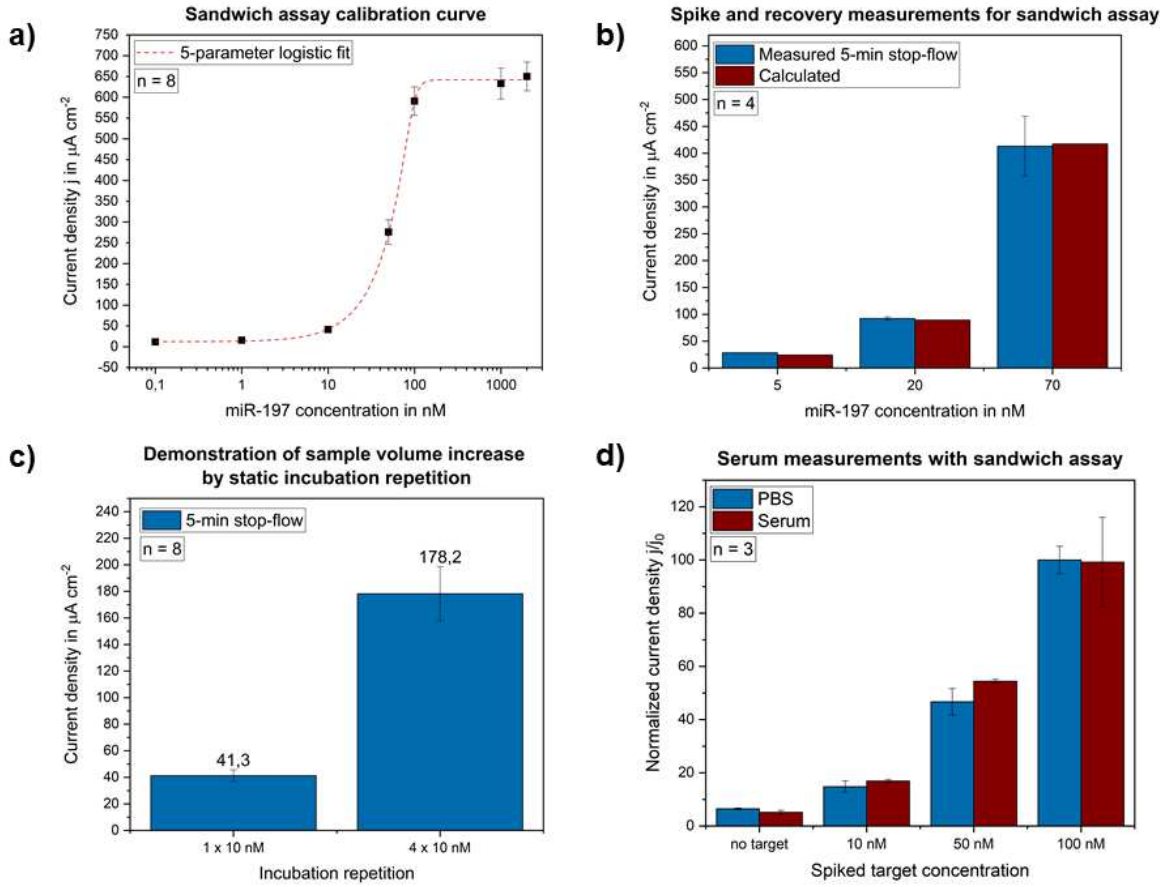

Figure 3: (a) *Standard curve obtained with the sandwich assay format.* ***(b)*** *Spike and recovery measurements performed for 5, 20, and 70 nM of target miRNA concentration. Obtained recovery yields are 85%, 96.6% and 101%, respectively.* ***(c)*** *Incubation repetition of a 10 nM concentrated sample for 4 successive times. By this, more sample volume is introduced to the microchannel and a higher signal response is obtained.* ***(d)*** *Comparison of the measurement results between serum and PBS, using signals obtained with 5-min stop-flow protocol. Here, 10, 50 and 100 nM of target miRNA-197 are spiked in almost undiluted serum (upon the addition of RNase-inhibitor). Error bars represent ±SD. For all measurements, the inter-assay CVs are below 15%, except the 100 nM measurement in serum with a CV of 17%.*

3.3. Comparison of sensitivity and dynamic range for competitive and sandwich assays

The performance of the sandwich assay is shown to be better than the competitive assay with respect to its sensitivity. Although the sensitivity of the competitive assay can be improved in a constrained way by reducing the concentration of the CCP, even for 10 nM CCP, it is not possible to achieve the sensitivity of the sandwich assay (4.05 nM for the competitive and 1.28 nM for the sandwich assay). Moreover, the signal response acquired with 10 nM CCP competitive assay is considerably lower when compared to the signal response of the sandwich

format. Lower signals can present a drawback for systems with higher noise or background signal levels.

On the other hand, competitive assay offers a broader dynamic range compared to the sandwich assay, when 50 and 100 nM CCP are used. This broadening is not due to an enhancement in the sensitivity (i.e. LOD), but due to an improvement in the ULOD. In a sandwich assay, in order to improve the ULOD, the amount of the immobilized capture DNA probes in the microchannel should be increased, which requires the extension of the cross-section of the microchannel.

The difference in the performance of sandwich and competitive assays could stem from the thermodynamic effects on oligonucleotide hybridization, with the assumption of the nearest-neighbor model. Here, the change in Gibbs free energy (ΔG) is of concern, which gives an understanding of the stability of the formed DNA-RNA duplex (Sugimoto et al., 1995). The design of the competitive capture probe and the sandwich capture and detection probes are different in the way that the sandwich probes are half the size of the competitive probe. Here, in the sandwich assay, the target hybridization takes place in two steps, in which the initiation ΔG takes different values than of the competitive assay hybridization. Moreover, in the sandwich assay, the dangling ends of the oligonucleotides play a role in the ΔG (Wetmur and Fresco, 1991). These effects might play a role in the stability of the formed DNA-RNA complex in both assays, and therefore, might explain the experimentally seen performance difference for both assay structures.

Table 2: *Overview of the calculated LOD and ULOD of the competitive and sandwich assays for miRNA-197 detection.*

	LOD in nM	**Dynamic range (LOD-ULOD) in nM**
100 nM CCP competitive assay	8.17	8.17-1,960
50 nM CCP competitive assay	5.89	5.89-651
50 nM CCP competitive assay with competitive advantage	4.51	4.51-430
10 nM CCP competitive assay	4.05	4.05-84
Sandwich assay	1.28	1.28-87

3.4. Selectivity of the competitive assay

The selectivity of both assay formats is investigated by replacing the target miRNA-197 in the incubated sample solutions with oligonucleotides of partially-matching or non-matching sequences. Partially-matching sequences include single-base terminal mismatch (1b-TMM), single-base central mismatch (1b-CMM) and three-base mismatch (3bMM) sequences. As a non-matching sequence, miRNA-19b is chosen.

In order to evaluate the selectivity of the competitive assay, 50 nM CCP and 2 µM of the alternative, partly or non-matching, oligonucleotide sequences are used. From the standard curve presented in figure 2c, it can be seen that the introduction of a sample with 2 µM target miRNA-197 hinders the binding of the competitor miRNA-197 to the capture probes, and the attained signal diminishes. Ideally, in a competitive assay, the replacement of the miRNA-197 should not cause any diminution in the signal, as partially- or non- matching sequences should not bind to the capture DNA probes.

For an easier comparison, the results of the selectivity tests, which are given in figure 4a, are normalized with respect to the blank signal, where no target miRNA is present. From figure 4a, it can be seen that the introduction of 1b-TMM causes a signal reduction of 97%, which is the same as the signal reduction caused by miR-197. Introduction of 1b-CMM, 3bMM and miR-19b result in a signal reduction of 89%, 83% and 5%, respectively. That suggests, the competitive assay shows very good selectivity against a non-matching sequence, very poor selectivity against 1b-CMM and 3bMM and no selectivity against 1b-TMM. This can be supported by applying unpaired parametric t-test to the means of the selectivity data. The means of the signal responses obtained by miR-197 and 1b-TMM show no significant difference when analyzed with a $*p < 0.05$, indicating no selectivity against 1b-TMM. Similarly, means of miR-19b and no target condition show no significant difference at $*p < 0.05$, which demonstrates very good selectivity against miR-19b.

3.5. Selectivity of the sandwich assay

Similar to the competitive format, for the sandwich assay, 2 µM target miRNA-197, which saturates the binding probes in the microchannel regarding the calibration curve presented in figure 3a, is replaced with 2 µM of the alternative oligonucleotide sequence in the incubation protocol. Ideally, introducing an oligonucleotide sequence other than miR-197 should not result in a signal, as this oligonucleotide should not bind either to the primary capture or the secondary detection DNA probes.

The results, as presented in figure 4b, are normalized with respect to the signal response obtained by the incubation of 2 µM target miRNA-197. From figure 4b, it is seen that the introduction of the non-matching sequence miR-19b yields almost no signal response (2%), which is comparable to the introduction of no target miRNA (2%). The signal responses obtained by the introduction of 3bMM, 1b-CMM and 1b-TMM sequences correspond to 6%, 30% and 44% of the signal obtained from miR-197, respectively. These indicate very good selectivity against 3bMM and non-matching miRNA sequences, whereas the selectivity against single-base mismatch sequences are only satisfactory. Still, when the means of partially- and non-matching sequences are compared with the mean of miR-197 by using an unpaired parametric t-test, for a high significance level of $******p < 0.0001$, they are found to be significantly different. Therefore, it is possible to conclude that the sandwich assay shows clear distinction towards single-base mismatch sequences.

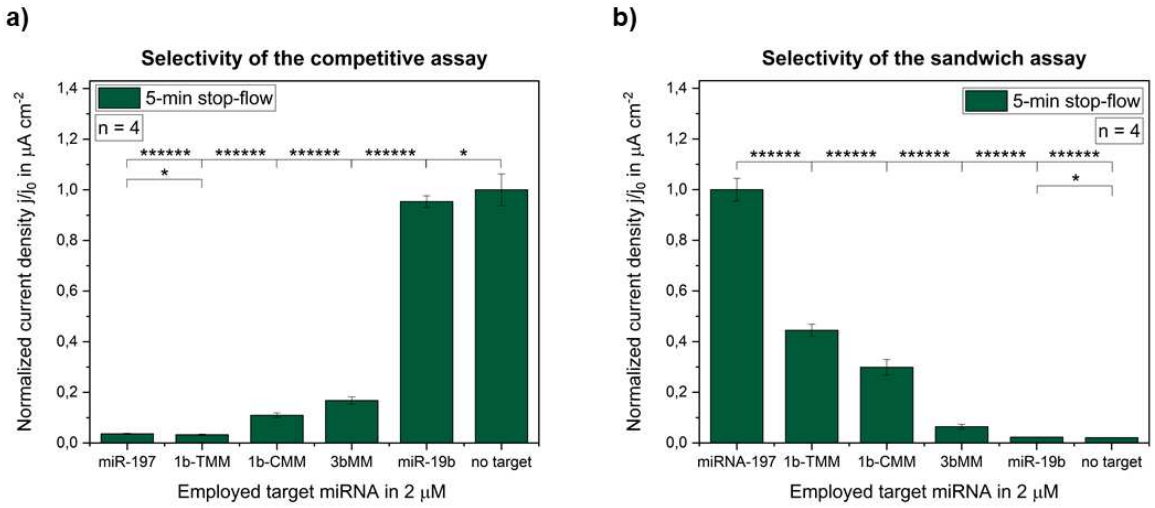

Figure 4: *Selectivity data obtained with 5-min stop-flow protocol for* ***(a)*** *competitive and* ***(b)*** *sandwich assays. Error bars indicate ± SD and inter-assay CV for all measurement points is below 15%. Means are analyzed by using an unpaired parametric t-test, with significance levels *p < 0.05 and ******p < 0.0001.*

3.6. Comparison of selectivity for competitor and sandwich assays

Sandwich assay has a better performance than the competitor one in terms of selectivity. Competitive assay shows no selectivity against 1b-TMM and very poor selectivity against 1b-CMM and 3bMM sequences. On the contrary, sandwich format demonstrates very good selectivity against 3bMM and, although not perfect, considerable discrimination against 1b-TMM and 1b-CMM sequences. Both assays prove very good discrimination against the non-matching sequence, miR-19b.

One interesting issue is that the capture probes for competitive assays, described in the literature, show usually better discrimination against single-base MM sequences, whereas, in this work, the competitive assay demonstrates almost no selectivity against even 3bMM. This might be due to the sequence of the chosen oligonucleotide probes, as the stability of the oligonucleotide duplex is strongly dependent on the oligonucleotide sequence and the mismatch positions (Davis and Znosko, 2007; SantaLucia and Hicks, 2004). Therefore, for competitive assays, the capture probe design might be more important compared to the sandwich assays and more stringent conditions might be necessary to enable for a better discrimination against few-base MM sequences.

3.7. Precision / Reproducibility

The measurements presented in this work are repeated three, four or eight times (n=3, n=4 and n=8, respectively), where the overall inter-assay CV for all measurements is below 15%. Only for the 100 nM-spiked serum measurement, the CV lies below 17%. No significant difference is observed between the competitive and sandwich assay in this regard. These results points to good reproducibility for both proposed assay formats as well as for the employed electrochemical biosensor platform.

3.8. Comparison of simplicity and handling

In the competitive assay, the balance between the capture DNA probes and the competitor miRNA is of crucial importance to the competition performance. The concentration of these key elements must be carefully optimized in order to realize a robust and sensitive assay. As this optimization process requires significant effort in the design and development of the assay procedure, in terms of simplicity, competitive format poses a disadvantage compared to the sandwich format.

In the sandwich format, the immobilization area should be packed with the possible highest density of capture and detection probes in order to improve the assay performance. Here, one concern is the possible unintended cross-bindings of the capture and detection probes, which should be carefully investigated during the assay development stages.

Parameters such as the sample volume or the hybridization medium (e.g. if the hybridization is realized in a solution outside the sensor environment where the diffusion of components are facilitated via shaking, etc.) might play a significant role for both assay formats. Yet, in terms of the assay development procedure, a change in such parameters may need an extensive re-optimization of the competitive assay components, which is not desired for systems, for example, where scaling might be needed.

For a point-of-care system, parameters such as total assay time, sample-to-result time and the number of incubation steps are also of concern. These parameters for the competitive and sandwich assay formats designed for this work are summarized in Supplementary Information. The sample-to-result time required in the competitive format without competitive advantage is 30 minutes, whereas it is 45 minutes for the sandwich assay. As a shorter sample-to-result time is more appealing to the end user, using competitive assay here is of advantage.

4. CONCLUSION and OUTLOOK

Here, we introduced, for the first time, a disposable electrochemical microfluidic biosensor for the on-site detection of miRNA-197 (a potential tumor biomarker) in undiluted human serum samples, requiring only very low sample volumes. For this purpose, the impact of two different (sandwich and competitive) assay formats on the electrochemical detection of miRNAs is studied. While competitive assay appears to have a broader dynamic range than the sandwich format, sandwich assay shows superior performance in terms of sensitivity and selectivity, possible due to the formation of a more stable DNA-RNA complex than in the case of competitive assay. There is no observable difference between both assays in terms of reproducibility.

The user should decide on the assay format by taking assay performance parameters into account and design the proper assay accordingly. In the case of miRNA detection, where the priority is to have a detection strategy which allows for extremely sensitive and selective measurements, it might be advantageous to use a sandwich assay.

For both assay formats presented in this work, the sensitivity should be further enhanced to be able to quantitatively measure miRNA levels in clinical samples. Therefore, different strategies,

including the implementation of secondary signal amplification mechanisms, such as utilization of secondary antibodies or isothermal amplification, will be investigated in future. Moreover, in a sandwich assay, the sample volume can be increased by realizing dynamic incubation with the help of a microfluidic setup. Using a dynamic incubation, a significantly greater sample volume can be passed through the microchannel without changing the biosensor structure, therefore, increasing the system sensitivity drastically.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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HIGHLIGHTS

- The detection of miRNA-197 using a biosensor is reported for the first time.
- Sandwich and competitive miRNA assays are compared regarding their performance.
- Sandwich assay is more sensitive and proves higher selectivity against mismatches.
- The microfluidic biosensor allows to work with very-low sample volumes (580 nl).
- The electrochemical biosensor is able to measure miRNA-197 in undiluted serum.

Competing interests

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Hazal Kutluk: Methodology, Conceptualization, Validation, Formal Analysis, Investigation, Resources, Writing – Original Draft, Visualization. **Richard Bruch:** Methodology, Resources, Writing – Review & Editing, Supervision. **Gerald Urban:** Funding Acquisition, Supervision. **Can Dincer:** Conceptualization, Methodology, Resources, Writing – Review and Editing, Supervision, Project Administration, Funding Acquisition.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: