Analytica Chimica Acta 1121 (2020) 35–41

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

CRISPR-dCas9 powered impedimetric biosensor for label-free detection of circulating tumor DNAs

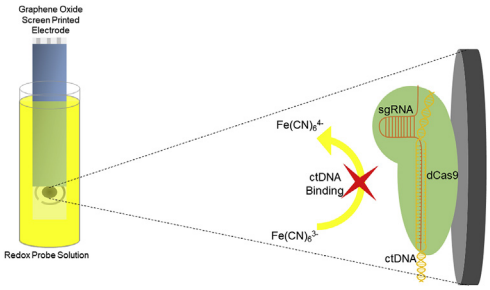
Zihni Onur Uygun [a, *], Levent Yeniay [b], Ferhan Gïrgïn Sağın [a]

[a] Ege University, Faculty of Medicine, Department of Medical Biochemistry, Bornova, 35100, İzmir, Turkey
[b] Ege University, Faculty of Medicine, Department of General Surgery, Bornova, 35100, İzmir, Turkey

HIGHLIGHTS

- First CRISPR/dCas biosensor for label-free circulating tumor DNA (ctDNA) detection.
- dCas9-sgRNA covalently immobilized on graphene oxide screen printed electrodes.
- Rapid, selective and accurate detection by electrochemical impedance spectroscopy.
- Promising form of liquid biopsy with CRISPR/Cas9 powered biosensor system for ctDNA.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 January 2020
Received in revised form
29 March 2020
Accepted 2 April 2020
Available online 16 April 2020

Keywords:
Impedimetric biosensor
Circulating tumor DNA
Chronoimpedance
CRISPR/Cas9 technology
Deactivated Cas9
Label-free analysis
Electrochemical impedance spectroscopy
Liquid biopsy
Cancer diagnosis

ABSTRACT

Label-free biosensors which can be integrated into lab-on-a-chip platforms have the advantage of using small volumes for rapid and inexpensive measurements contrary to label-based technologies which are often more costly and time-consuming. In this study, graphene oxide screen printed electrodes (GPHOXE) were modified by deactivated Cas9 (dCas9) proteins and synthetic guide RNA (sgRNA) as the biorecognition receptor for label-free detection of circulating tumor DNAs (ctDNA). This was achieved by detection of a tumor related mutation (PIK3CA exon 9 mutation) via sequence-specific recognition followed by electrochemical impedance spectroscopy (EIS) analysis. The biosensor showed high specificity as there was no impedance signal for other ctDNA sequences, even the single nucleotide mismatch. dCas9-sgRNA modified biosensor demonstrated linear detection limits between 2 and 20 nM for 120 bp ctDNA's in 40 s. The calibration curve showed good linearity, LOD was calculated as 0.65 nM and LOQ was calculated as 1.92 nM. Selectivity and repeatability studies were carried out in real blood samples and the recovery was higher than 96%. In conclusion, dCas9-sgRNA was effectively immobilized and optimized on GPHOXE as the selective biorecognition receptor of this ultrafast impedimetric biosensor. The CRISPR-dCas9 powered impedimetric system showed good selectivity, high repeatability and good recovery properties. This is the first literature to report the use of CRISPR/Cas technology as a label-free tool that can be used in an impedimetric system for detection of ctDNA's.

1. Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide and the number of patients diagnosed with cancer has increased by 70% in the last 20 years [1]. Early diagnosis and

* Corresponding author. +902323903699.
E-mail address: onur.uygun@ege.edu.tr (Z.O. Uygun).

https://doi.org/10.1016/j.aca.2020.04.009
0003-2670/

appropriate treatment of cancer requires data on the morphological and genetic characteristics of the cancer tissue and cells. However, except the hematopoietic system cancers or skin tumors, the malignant cells are obtained through tissue biopsies which are mostly invasive, non-reproducible and time consuming. Besides, the high-cost and high-morbidity, and even stimulation of cancer progressions and metastasis related to the procedure add to the disadvantages. Some cancerous lesions may also be overlooked by tissue biopsy methods that can only examine a portion of the tumor mass [2]. Therefore, systemic cancer therapies may be unsuccessful due to intratumoral heterogeneity, which is difficult to capture by tissue biopsy [3].

Liquid biopsy is a revolutionary technique which provides a noninvasive approach to tumor molecular profiling without the need to perform tissue biopsy. It is the analysis of blood samples of cancer patients and aims to measure biological markers in the bloodstream such as circulating tumor cells (CTC), circulating tumor DNA (ctDNA) and other extracellular free nucleic acids and exosomes [4]. Extracellular nucleic acids are small nucleic acids associated with cancer cells. Compared to CTCs, ctDNA is easier to isolate and more sensitive for detection [5]. These DNA fragments in the bloodstream may originate during cellular cycling, such as stimulation of lymphocytes or by death of apoptotic and necrotic cells [6]. Under normal physiological conditions, apoptotic and necrotic cells are cleared by phagocytes, and extracellular DNA levels appear to be relatively low, but it is known that this cleaning mechanism is not effective in tumor mass [7]. Thus, ctDNA carry the driver mutations causing metastases and provide an accurate molecular profiling of the tumor. It is clear that learning about the genomics of a particular cancer through a tube of blood drawn from the patient has many advantages over the current 'gold standard' of tissue biopsy. Therefore, we aimed to develop a new biosensor system to detect ctDNA as a cancer biomarker.

Biosensor systems use biological molecules such as enzymes, DNAs, peptides, RNAs, as biorecognition receptors [8–12] and convert a biological response into an electrical, electrochemical, magnetic, optical, etc. signal. The newly developed biosensor of this study targets a specific genetic sequence in the DNA (a specific mutation that plays a role in cancer development) and involves the innovative use of the CRISPR/dCas9, a protein-RNA complex as the biorecognition receptor agent that would specifically bind to this sequence. Previous methods were based on the DNA-DNA interaction or field effected transistors. In this study, we proposed a label-free and impedimetric method to detect ctDNA in seconds. Moreover, our method is more selective and accurate by comparing other methods. A modified version of Cas9 without endonuclease activity was used as biorecognition receptor complex element [13–19]. These catalytically inactive Cas9 proteins (deactivated Cas9, dCas9) do not interfere with DNA hydrolysis, they bind only to the specific DNA region without interrupting the target DNA, physically interfering with the gene transcription process [20]. Thus, this biorecognition complex (dCas9-sgRNA complex) in our biosensor will specifically bind to ctDNAs in the plasma carrying the tumor-specific genetic mutations. Moreover, apart from the other DNA biosensors and sensor, we did not break the helix to form single stranded DNA, we just measured the double stranded DNA instead. That was an advantageous step for us to remove the former step for DNA helix breakdown. Nanomaterials are being used in biosensor technologies to increase sensitivity by improving surface area of the electrode, conductivity and to form a unique immobilization material for biomolecules. Therefore, we used graphene oxide electrodes (GPHOXE) for an easy immobilization procedure as well as to increase the repeatability of the immobilization procedure and the sensitivity. Graphene oxides have oxidized carbonyl groups and these groups were activated by *N*- (3-dimethylaminopropyl) −*N*′-ethylenediaminecarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Cas9 proteins and sgRNA's (biorecognition receptor complex) were then covalently immobilized on graphene oxide screen printed electrodes. This immobilization and the ctDNA detection were investigated by the electrochemical impedance spectroscopy, a good method to monitor electrode surface modifications and characteristics.

2. Materials and methods

2.1. Chemicals and equipment

dCas9 proteins were obtained from Applied Biological Materials (ABM) Inc. (Richmond, BC, Canada), sgRNA sequences were obtained from Synthego Corp. (CA, USA) and ctDNA sequences were obtained from Metabion International AG (Germany). Other chemicals were obtained from Sigma Aldrich (USA). Graphene oxide screen printed electrodes (DRP-110GPHOX) were obtained from Metrohm Dropsens (Switzerland). PalmSens 3 potentiostate system was used for electrochemical techniques and PSTrace 5.6 was used as electrochemical interface software. Atomic Force Microscope data were obtained from ezAFM from Nanomagnetics (UK).

2.2. Method

2.2.1. Sample collection and patient selections

All consumable plastic or glass equipments were obtained as DNAase free, RNAase free and endotoxin-free types. This study was approved by Ege University, School of Medicine, Clinical Research Ethical Committee (Approval number of 16-12.1/48). Breast cancer patients, who have been newly diagnosed without being treated with any drug or surgical operations, were chosen for this study. Blood samples were collected in sterile tubes including EDTA and stored in −80 °C until use. PI3K Mutation Test Kit from Qiagen© (Qiagen, Hilden, Germany, http://www.qiagen.com) was used, which contained one control assay and three mutations assays to be run in a qPCR assay. The qPCR was performed on ctDNA from all breast carcinoma samples using the Roche® LC 480 instrument [21].

2.2.2. Biosensor modification steps

Electrochemical impedance spectroscopy (EIS) was used as the basic measurement and surface characterization method. In our study, surface electron transfer resistance was measured by performing EIS in pH = 7 phosphate buffer containing solution 5 mM $Fe(CN)_6^{3-/4-}$ as a pair of redox probe and 10 mM KCl. EIS settings were set as 180 mV potential, which was applied to the electrode as DC potential, and impedance measurement was performed by frequency scanning between 2000 and 0.05 Hz. Cyclic voltammetry

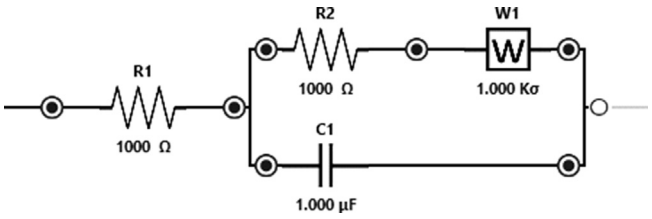


Fig. 1. Randles circuit model for electrochemical impedance spectroscopy fitting. R1 is the electron transfer resistance between the working and counter electrode of the redox probe solution, R2 is the electron transfer resistance of the working electrode surface that is the signal we used to measure ctDNA binding to the electrode by EIS, W1 is Warburg impedance that shows redox probe transfer resistance towards the surface of the working electrode, Q1 is the constant phase element that shows electrical double layer capacitance of the electrode.

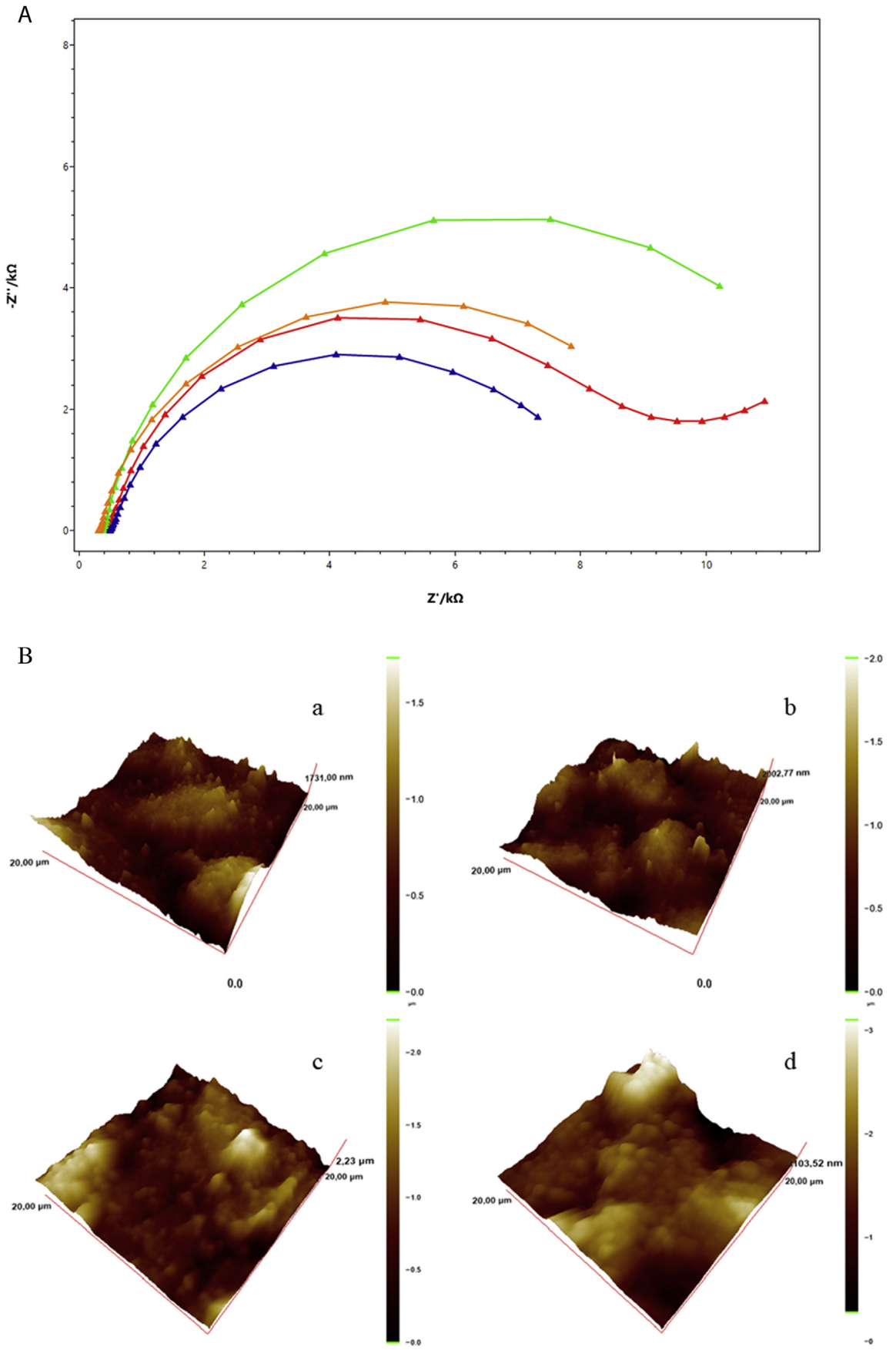


Fig. 2. A; Electrode modification steps demonstrated with impedance curves from electrochemical impedance spectroscopy data. Red: GPHOXE, Blue: GPHOXE-dCas9, Yellow: GPHOXE-dCas9-sgRNA, Green: GPHOXE-dCas9-gRNA-ctDNA. **B;** Atomic force microscopy images of the ctDNA electrode modification steps (a; GPHOXE, b; GPHOXE/dCas9, c; GPHOXE/dCas9/sgRNA, d; GPHOXE/dCas9/sgRNA/ctDNA).

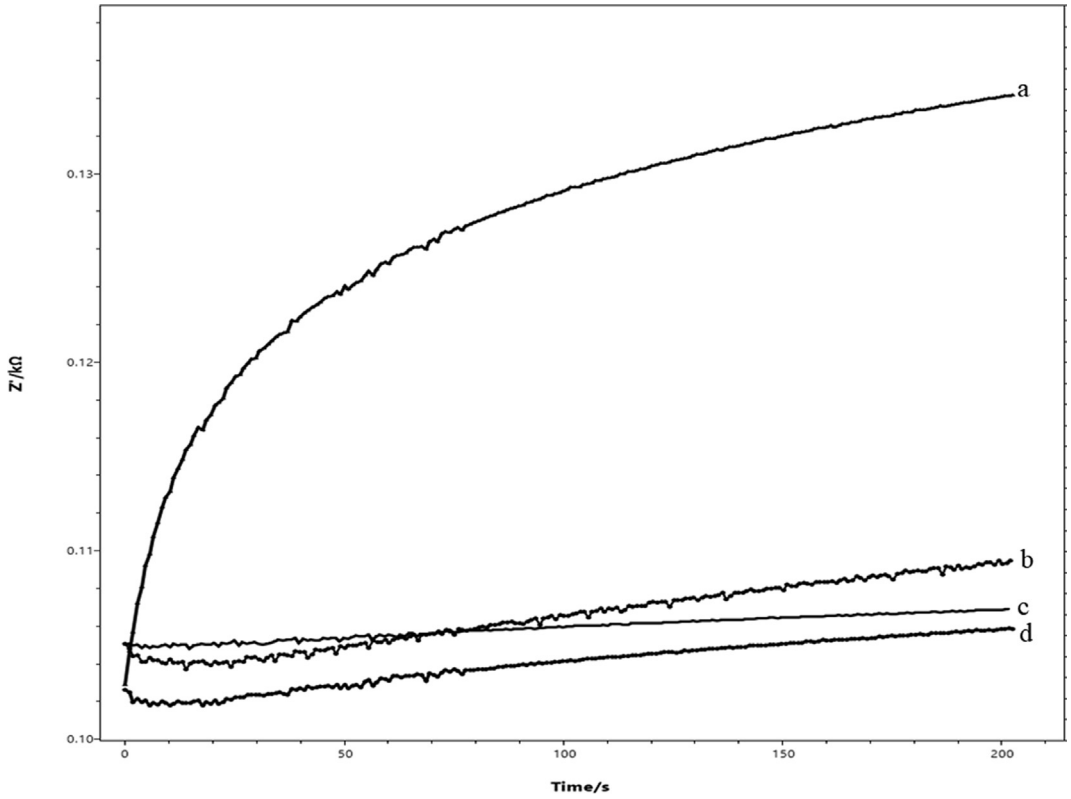


Fig. 3. Chronoimpedimetric ctDNA detection, a) 120 bp mutant ctDNA, b) single nucleotide mutation ctDNA, c) 120 bp wild-type ctDNA, d) non-DNA included sample. Chronoimpedimetric results of the other ctDNA sequences, even the single nucleotide mismatch, results in no impedance signal and thus indicate the high selectivity of the new CRISPR-dCas9 powered impedimetric biosensor.

(CV) was performed in the same redox probe. CV conditions were between −0.2 and 0.5 V at a scanning speed of 50 mV/s. Firstly, GPHOXE was pretreated to form more carbonyl groups by applying 1.6 V for 150 s in 2 M H_2SO_4 solution. Then the electrode was washed, EIS was performed for baseline (GPHOXE). Afterwards a solution containing 1:4 (10 mM: 40 mM) of *N*- (3-dimethylaminopropyl) −*N*′-ethylenediaminecarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) was dropped on the electrode and incubated for 1 h in room temperature to activate carboxyl groups to bind the biorecognition receptor covalently [22]. After 1-h, the electrode was washed with pure water and dried under gentle nitrogen stream. Then 20 μL of 500 nM of dCas9 protein was dropped on GPHOXE and incubated for 1 h in room temperature and environment was humidified to prevent evaporation of dropped dCas9 solution. After 1 h EIS was carried out to measure GPHOXE/dCas9 electrode modification. Then, 20 μL of 500 nM sgRNA was dropped on the GPHOXE/dCas9 electrode and incubated in 37^{O}C to modify electrode as GPHOXE/dCas9/sgRNA. According to the literature, sgRNA was chosen as follows to target our DNA sequence.

(sgRNA sequence (N: nucleotide): 5'NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU-3') [23]. Subsequently, electrode was washed to remove unbounded sgRNA and EIS was performed for GPHOXE/dCas9-sgRNA electrode. After all these modification steps, the ctDNA biosensor was prepared to monitor ctDNA. All impedance curves were fitted to calculate electron transfer resistances by using Randles Circuit model [24] shown in Fig. 1. Besides, all electrode modification steps were also investigated by Atomic Force Microscopy. AFM mod was chosen as Tapping Mode, resolution was 256 × 256 pixels, ACLA was used as cantilever, and vibration was 1 $V_{RMS.}$ All biosensor optimization steps were also carried out and explained in the supplementary file (Suppl. 1).

2.2.3. Biosensor performance tests

After the modification steps of the GPHOXE/dCas9-sgRNA modified electrode, ctDNA detections were performed. The target ctDNA sequence was chosen according to a study published in Nature Communications in 2016 that evaluated the genetic profiling of 2433 breast cancer case s [25]. In this study, 40.1% of the cases had PIK3CA mutations. Although somatic mutations in PIK3CA have been identified at different rates in different countries, the majority of mutations appear to be in the helical domain (exon 9) of PIK3CA. Therefore, we chose ctDNA sequence as; Exon 9: G1633A, C1616G, A1634C, G1624A. We consider these point mutations as ctDNA and calibration curve was performed at 37.5^{O}C for 3 min by dropping 2 nM−20 nM 120 bp ctDNA on GPHOXE/dCas9-sgRNA electrode. The electrode performance temperature was set between 36 and 38 ^{O}C. The pH was used as the same as blood, which is 7.4. LOD and LOQ were calculated by using the equations as follows; LOD = 3.3 (S/m) and LOQ = 10 (S/m). S is the standard deviation of the 2 nM concentration signal of five repeated detections and m is the slope of the calibration curve. Reproducibility was tested by forming 9 calibration curves to assess their coefficient of regression and slope of calibration curves. Repeatability was tested by monitoring two different samples for three times by six different biosensors. Recovery tests were carried out by adding 15 nM ctDNA into the samples and performed by the biosensor.

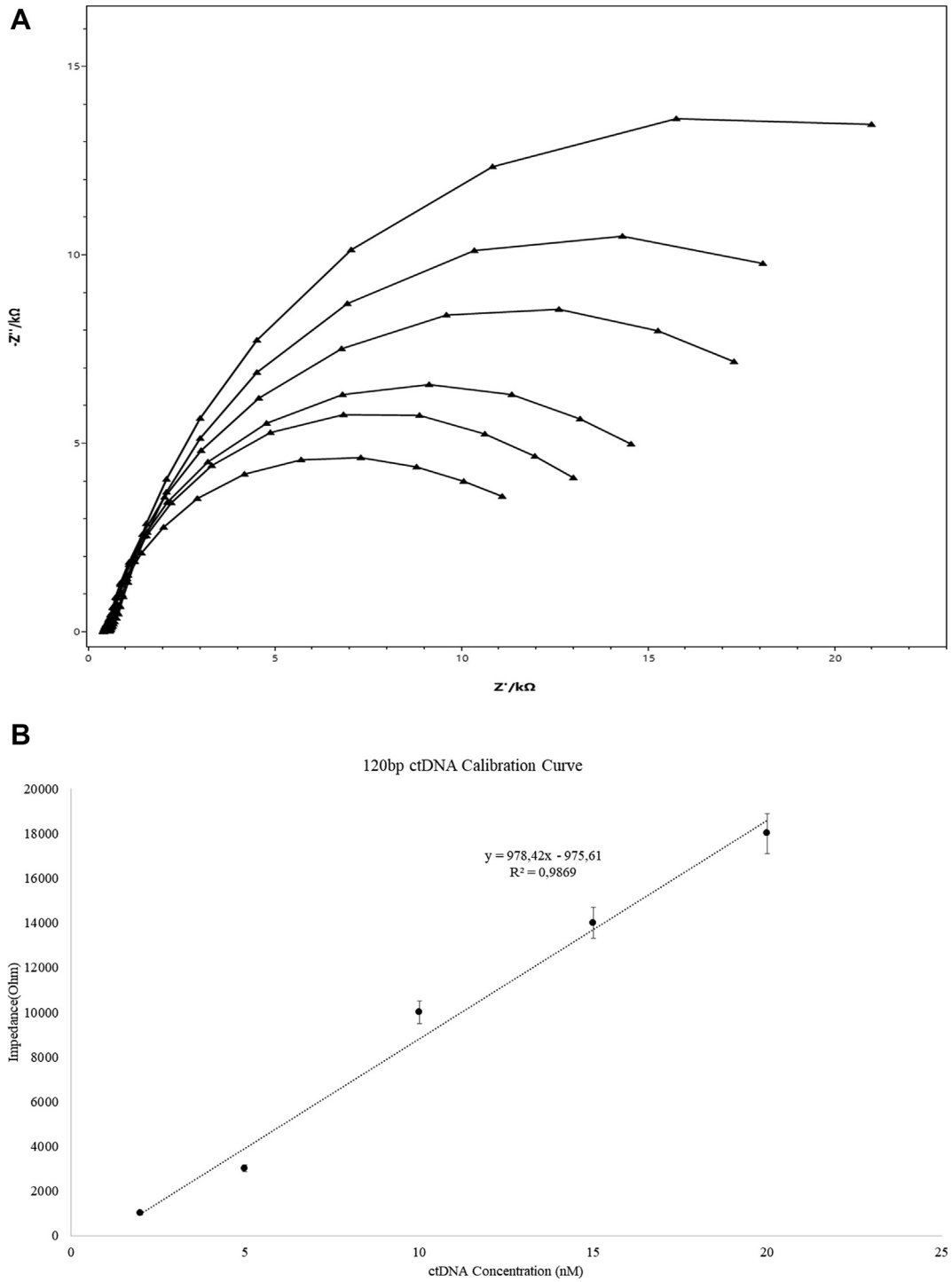


Fig. 4. ctDNA biosensor calibration curves from 0, 2.5, 5, 10, 15, 20 nM, A; EIS spectrums, **B;** Calibration curve, x-axis represents the ctDNA concentration, y-axis represents the impedance results (ohm).

3. Results and discussion

3.1. Electrode modifications

Before the detection of the ctDNA in blood was carried out by the new CRISPR-dCas9 powered impedimetric biosensor system, electrode modification steps were performed first. Fig. 2 shows these data from electrochemical impedance spectroscopy (Fig. 2A) and atomic force microscopy (Fig. 2B).

The activated electrode was covalently modified by dCas9 and the result is a decreased impedance curve which was shown by blue line. This is the result of the decreased number of carboxyl groups freely available to repel redox probe from the surface [11] and the positively charged dCas9 proteins [26] attracts redox probe to decrease electron transfer resistance. Due to the same reason, the Warburg impedance no more existed and mass transfer resistance lost its dominancy. On the contrary, when sgRNA modification and ctDNA binding took place successfully, an obvious increase in

Table 1
Performance characteristics of the ctDNA biosensor compared with other biosensors (Abbreviations: CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats, Cas: Caspase, SWV: square wave voltammetry, LSPR: localized surface plasmon resonance, PNA: peptide nucleic acids, AuNPs: gold nanoparticles, PIn6COOH:polyindole-6-carboxylic acid, ST: scrub typhus and SFTS: severe fever with thrombocytopenia syndrome, SMR: single microring resonator, gFET: graphene-based field-effect transistor).

	Our Study	[27]	[28]	[29]	[19]	[18]
Target	PIK3CA(Exon 9)	PIK3CA	PIK3CA	PIK3CA	ST, SFTS	Duchenne muscular dystrophy
Electrochemical Method	EIS	SWV	LSPR	EIS	SMR	FET
Biorecognition Receptor	CRISPR-dCas9	PNA-AuNPs	PNA-AuNPs	PIn6COOH/MoS_2	CRISPR-dCas9	gFET- CRISPR–Cas9
Detection Time	40 Seconds	30 Minutes	30 Minutes	–	–	15 Minutes
Linear Range	2–20 nM	20-10000 fM	50-3200 fM	1.0×10^{-16} to 1.0×10^{-11} M	–	0–1800 ng
R^2	0.9869 ± 0.0042	0.9915	0.99	–	–	–
LOD	0.65 nM	10 fM	50 fM	1.5×10^{-17}M	0.54 aM (ST) 0.63 aM (SFTS)	1.7 fM
LOQ	1.92 nM	–	–	–	–	–
Repeatability	(Sample 1: 9 nM) 8.43 ± 0.21 nM Sample 2: 17 nM 16.83 ± 0.35 nM	1000 fM 5.3%	1.0×10^{-16} M 5.33%	–	–	–
Recovery	(15 nM) 96.53% 14.48 ± 0.16 nM	(50 fM) 104.2% 52.11 ± 1.09	–	–	–	–
Reproducibility	R^2 = 0.9869 ± 0.0042 m = 978.42 ± 0.11	RSD of 5.3%	–	–	–	–

electron transfer resistance is detected (yellow and green curves, respectively). As can be seen, there are differences in surface thickness through each modification layer from a to d which indicate a successful modification process.

Surface modification histograms that also support the modification process and show thickness of the surface are shown in the supplementary data (Suppl. 1.).

3.2. Biosensor performance tests

The ctDNA sequence in real samples is over 120 bp in general [5], thus we obtained 120bp ctDNA fragments in different concentrations. These were added on to the GPHOXE-dCas9-sgRNA electrode for chronoimpedimetric detection and to observe the detection time. Chronoimpedance measurement was performed to monitor the saturation of the biosensor surface by ctDNA. The first linear increase was observed in 40 s and biosensor detection time was chosen as 40 s. Chronoimpedance conditions were obtained from our previous study [22]. The results were shown in Fig. 3(Fig. 3.).

As can be seen in Fig. 3, the ctDNA binding (a) increases until 40 s and the curve turns to a plateau in 200 s. After identifying the detection time, the linear calibration range was determined with 120bp ctDNA standards. Thus, the concentration-binding time (time of ctDNA binding to the electrode) versus the EIS spectrums were obtained (Fig. 4A) and EIS data fitting was carried out by the circuit model shown in Fig. 1. GPHOXE-dCas9-gRNA EIS signal was taken as baseline ($Resistance_{Blank}$, R_B), and the impedance value obtained for binding of ctDNA to the dCas9-gRNA complex as $R_{[ctDNA]}$. Accordingly, the standard concentration was plotted on the graph by calculating the impedance $\Delta R_{[ctDNA]} = R_{[ctDNA]} - R_B$ (Fig. 4B). Another point to note here is that the amount of ctDNA is calculated as nM for 40 s rather than calculated in nM. Afterwards, nine calibration curves were prepared to assess linearity and reproducibility. As can be seen in Fig. 4A and B, good linearity was observed between 0 and 20 nM after which the linearity decreased.

To perform selectivity studies of the new CRISPR-dCas9 powered impedimetric biosensor, wild type DNA sequence (healthy subjects' sequence) was used as the target ctDNA sequence. For the GPHOXE/dCas9, sgRNA binding was detected to be normal but no non-complementary PIK3 sequence was bonded to the sgRNA sequence. This can be monitored by the absence of increase in the impedance curve in Supplementary Fig. 2 (the black curve is GPHOXE-dCas9-gRNA and the light green color is the impedance curve of the PIK3 sequence). This proves the high selectivity performance of the new biosensor system (Suppl. Fig. 2).

The detection limits of the biosensor were tested by calculating LOD and LOQ. LOD was calculated as 0.65 nM, LOQ was calculated as 1.92 nM. LOQ is close to the beginning point of calibration curve, this represents the linearity starts at 2 nM level. This was proved both experimentally and mathematically.

Reproducibility was tested by forming 9 calibration curves and assessing the regression coefficient (R^2) and slope of calibration curve (m) of these 9 calibration curves'. Average R^2 is calculated with standard deviation as 0.9863 ± 0.0042 and average of the slope of calibration curve with standard deviation is found to be 978.42 ± 0.11.

For the real blood sample analysis, we added 15 nM 120 bp standards to samples to measure recovery and real blood sample analysis. Our biosensor reached 96.53% recovery potential (mean: 14.48 ± 0.16 nM). Repeatability tests were carried out in 2 different blood samples, and standards of 9 nM and 17 nM were added. Each sample was tested three times with three different biosensors. First sample was determines as 8.43 ± 0.21 nM while the second sample was found to be 16.83 ± 0.35 nM. Whole results are given in Table 1 (Table 1). The results showed that ctDNA levels can be detected with more than 96% accuracy. Our sensor has narrow linear range and higher LOD but, these are the results of the label free detections. On the other hand, biosensor provides label-free detection, high specificity and rapid response to the target. Moreover, ctDNA sample preparation does not need a denaturation of the DNAs.

4. Conclusion

This is the first published work on a label-free, CRISPR-powered impedimetric biosensor for the detection of PIK3CA exon 9 mutations in ctDNA's. The novelty of our work resides in the fact that we used CRISPR/Cas technology and successfully modified GPHOXE with dCas9-sgRNA. The modification process resulted with good

conductivity and surface area for repeatable modification layers. The detection of ctDNA was further carried out by adding standards to real blood samples. The high selectivity (no increase in impedance curves with other non-complementary DNA) and the high accuracy (more than 96%) as well as the ultra-fast detection time (40 s) indicate that CRISPR/Cas9 powered biosensor system is promising to detect the presence of ctDNA as a novel form of liquid biopsy. The possible application of the technique with other important mutations in a multiplex system may be a promising area in early diagnosis of cancers.

Declaration of competing interest

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.

CRediT authorship contribution statement

Zihni Onur Uygun: Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Levent Yeniay:** Conceptualization. **Ferhan Gİrgİn Sağın:** Project administration, Supervision, Funding acquisition.

Acknowledgements

This study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK-1001) with the project number of 217S353.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.04.009.

References

[1] B.W. Stewart, C. Wild, International Agency for Research on Cancer., World Health Organization, International Agency for Research on Cancer, 2014. World cancer report 2014.
[2] P.H. Abbosh, J.E. Rosenberg, E.R. Plimack, Circulating biomarkers to guide systemic therapy for urothelial carcinoma, Urol. Oncol. Semin. Orig. Investig. 34 (2016) 502–509, https://doi.org/10.1016/j.urolonc.2016.08.019.
[3] E. Heitzer, P. Ulz, J.B. Geigl, Circulating tumor DNA as a liquid biopsy for cancer, Clin. Chem. 61 (2015) 112–123, https://doi.org/10.1373/clinchem.2014.222679.
[4] E. Crowley, F. Di Nicolantonio, F. Loupakis, A. Bardelli, Liquid biopsy: monitoring cancer-genetics in the blood, Nat. Rev. Clin. Oncol. 10 (2013), https://doi.org/10.1038/nrclinonc.2013.110.
[5] L. De Mattos-Arruda, C. Caldas, Cell-free circulating tumour DNA as a liquid biopsy in breast cancer, Mol. Oncol. 10 (2016) 464–474, https://doi.org/10.1016/j.molonc.2015.12.001.
[6] Z. Qin, V.A. Ljubimov, C. Zhou, Y. Tong, J. Liang, Cell-free circulating tumor DNA in cancer, Chin. J. Canc. 35 (2016) 36, https://doi.org/10.1186/s40880-016-0092-4.
[7] J.V. Canzoniero, B.H. Park, Use of cell free DNA in breast oncology, Biochim. Biophys. Acta Rev. Canc 1865 (2016) 266–274, https://doi.org/10.1016/j.bbcan.2016.03.006.
[8] T.H. Degefa, J. Kwak, Electrochemical impedance sensing of DNA at PNA self assembled monolayer, J. Electroanal. Chem. 612 (2008) 37–41, https://doi.org/10.1016/j.jelechem.2007.09.004.
[9] C.R. Lowe, Biosensors, Trends Biotechnol. 2 (1984) 59–65, https://doi.org/10.1016/0167-7799(84)90011-8.
[10] N.J. Ronkainen, H.B. Halsall, W.R. Heineman, Electrochemical biosensors, Chem. Soc. Rev. 39 (2010) 1747, https://doi.org/10.1039/b714449k.
[11] H. Deniz, Z. Onur, Impedimetric biosensors for label-free and enzymless detection, in: State Art Biosens. - Gen. Asp., InTech, 2013, https://doi.org/10.5772/52169.
[12] X. Zhang, H. Ju, J. Wang, Electrochemical Sensors, Biosensors and Their Biomedical Applications, 2008, https://doi.org/10.1017/CBO9781107415324.004.
[13] Y. Li, S. Li, J. Wang, G. Liu, CRISPR/Cas systems towards next-generation biosensing, Trends Biotechnol. 37 (2019) 730–743, https://doi.org/10.1016/j.tibtech.2018.12.005.
[14] C. Myhrvold, C.A. Freije, J.S. Gootenberg, O.O. Abudayyeh, H.C. Metsky, A.F. Durbin, M.J. Kellner, A.L. Tan, L.M. Paul, L.A. Parham, K.F. Garcia, K.G. Barnes, B. Chak, A. Mondini, M.L. Nogueira, S. Isern, S.F. Michael, I. Lorenzana, N.L. Yozwiak, B.L. MacInnis, I. Bosch, L. Gehrke, F. Zhang, P.C. Sabeti, Field-deployable viral diagnostics using CRISPR-Cas13, Science (80-.) 360 (2018) 444–448, https://doi.org/10.1126/science.aas8836.
[15] R. Bruch, G.A. Urban, C. Dincer, CRISPR/Cas powered multiplexed biosensing, Trends Biotechnol. 37 (2019) 791–792, https://doi.org/10.1016/j.tibtech.2019.04.005.
[16] R. Bruch, G.A. Urban, C. Dincer, Unamplified gene sensing via Cas9 on graphene, Nat. Biomed. Eng. 3 (2019) 419–420, https://doi.org/10.1038/s41551-019-0413-4.
[17] J. Hu, M. Jiang, R. Liu, Y. Lv, Label-free CRISPR/Cas9 assay for site-specific nucleic acid detection, Anal. Chem. 91 (2019) 10870–10878, https://doi.org/10.1021/acs.analchem.9b02641.
[18] R. Hajian, S. Balderston, T. Tran, T. deBoer, J. Etienne, M. Sandhu, N.A. Wauford, J.-Y. Chung, J. Nokes, M. Athaiya, J. Paredes, R. Peytavi, B. Goldsmith, N. Murthy, I.M. Conboy, K. Aran, Detection of unamplified target genes via CRISPR–Cas9 immobilized on a graphene field-effect transistor, Nat. Biomed. Eng. 3 (2019) 427–437, https://doi.org/10.1038/s41551-019-0371-x.
[19] B. Koo, D. Kim, J. Kweon, C.E. Jin, S.-H. Kim, Y. Kim, Y. Shin, CRISPR/dCas9-mediated biosensor for detection of tick-borne diseases, Sensor. Actuator. B Chem. 273 (2018) 316–321, https://doi.org/10.1016/J.SNB.2018.06.069.
[20] D. Balboa, J. Weltner, S. Eurola, R. Trokovic, K. Wartiovaara, T. Otonkoski, Stem cell reports conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation, Stem Cell Reports 5 (2015) 448–459, https://doi.org/10.1016/j.stemcr.2015.08.001.
[21] I. Palimaru, A. Brügmann, M.K. Wium-Andersen, E. Nexo, B.S. Sorensen, Expression of PIK3CA, PTEN mRNA and PIK3CA mutations in primary breast cancer: association with lymph node metastases, SpringerPlus 2 (2013) 464, https://doi.org/10.1186/2193-1801-2-464.
[22] Z.O. Uygun, Ç. Şahin, M. Yılmaz, Y. Akçay, A. Akdemir, F. Sağın, Fullerene-PAMAM(G5) composite modified impedimetric biosensor to detect Fetuin-A in real blood samples, Anal. Biochem. 542 (2018) 11–15, https://doi.org/10.1016/J.AB.2017.11.007.
[23] S.B. Thyme, L. Akhmetova, T.G. Montague, E. Valen, A.F. Schier, Internal guide RNA interactions interfere with Cas9-mediated cleavage, Nat. Commun. 7 (2016) 11750, https://doi.org/10.1038/ncomms11750.
[24] Z.O. Uygun, H.D. Ertuğrul Uygun, A short footnote: circuit design for faradaic impedimetric sensors and biosensors, Sensor. Actuator. B Chem. 202 (2014) 448–453, https://doi.org/10.1016/j.snb.2014.05.029.
[25] B. Pereira, S.-F. Chin, O.M. Rueda, H.-K.M. Vollan, E. Provenzano, H.A. Bardwell, M. Pugh, L. Jones, R. Russell, S.-J. Sammut, D.W.Y. Tsui, B. Liu, S.-J. Dawson, J. Abraham, H. Northen, J.F. Peden, A. Mukherjee, G. Turashvili, A.R. Green, S. McKinney, A. Oloumi, S. Shah, N. Rosenfeld, L. Murphy, D.R. Bentley, I.O. Ellis, A. Purushotham, S.E. Pinder, A.-L. Børresen-Dale, H.M. Earl, P.D. Pharoah, M.T. Ross, S. Aparicio, C. Caldas, The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes, Nat. Commun. 7 (2016), 11479, https://doi.org/10.1038/ncomms11479.
[26] W. Sun, W. Ji, J.M. Hall, Q. Hu, C. Wang, C.L. Beisel, Z. Gu, Self-Assembled DNA nanoclews for the efficient delivery of CRISPR-cas9 for genome editing, Angew. Chem. Int. Ed. 54 (2015) 12029–12033, https://doi.org/10.1002/anie.201506030.
[27] C. Cai, Z. Guo, Y. Cao, W. Zhang, Y. Chen, A dual biomarker detection platform for quantitating circulating tumor DNA (ctDNA), Nanotheranostics 2 (2018) 12–20, https://doi.org/10.7150/ntno.22419.
[28] A.H. Nguyen, S.J. Sim, Nanoplasmonic biosensor: detection and amplification of dual bio-signatures of circulating tumor DNA, Biosens. Bioelectron. 67 (2015) 443–449, https://doi.org/10.1016/J.BIOS.2014.09.003.
[29] J. Yang, X. Yin, W. Zhang, Electrochemical determination of PIK3CA gene associated with breast cancer based on molybdenum disulfide nanosheet-supported poly(indole-6-carboxylic acid), Anal. Methods. 11 (2019) 157–162, https://doi.org/10.1039/C8AY02425A.