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Paper-based screen-printed electrode to detect miRNA-652 associated to triple-negative breast cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) is one of the most aggressive and lethal types of BC, affecting mostly young women and its diagnosis is difficult and requires invasive methods, such as tissue biopsy which is painful and expensive. However, nowadays liquid biopsy is emerging as a great tool for determining blood-circulating species associated to cancer and to its early diagnosis and prognosis. Among the species, the relevance of microRNAs (miRNAs) has been highlighted as promising biomarker, and miRNA-652 has been associated with TNBC as it promotes the growth and migration of cancer cells. In this work we designed and characterized a paper-based electrochemical device capable of recognizing and quantifying miRNA-652, as future tool for liquid biopsy in TNBC. The device consists of an AuNP-modified office paper-based screen-printed electrode customized with an anti-miRNA probe for the selective recognition of miRNA-652. All the experimental parameters have been carefully evaluated, and the platform allowed to detect miRNA-652 in standard solution and human serum down to 0.4 nM, with a satisfactory repeatability of about 6 and 3 % respectively. The selectivity presence of other miRNA sequences was satisfactory demonstrated. In addition, we demonstrated the effectiveness of pre-concentration of miRNA by coupling the office paper-based electrode with an external disk made by chromatographic paper: the detection limit has been improved of 10-fold without the use of complex/expensive procedures. The presented manuscript represents an important step towards the development of a non-invasive, sensitive and TNBC-specific diagnostic platform that could improve patients' prognosis and quality of life, ulteriorly improved with pre-concentering properties of frugal supports as the external chromatographic paper-based ones.

1. Introduction

Despite constant advances in the field of medicine, breast cancer (BC) continues representing the second most common and deadly type of malignancy among women. Its incidence has seen an alarming increase over the last forty years. In 2020, approximately 2.3 million new cases of BC were diagnosed globally, and there were approximately 700k deaths due to this disease [1]. In clinical terms, particular subtypes of BC are identified on the basis of histopathological appearance and the presence of hormone receptors and growth factors. In particular, the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are considered [2]. Gene expression analysis has made it possible to subdivide breast cancer into various molecular subtypes. These include subtypes with positive receptors (2-positive) and those with negative receptors (TNBC (Triple negative breast cancer)) [3]. TNBC is a highly aggressive form of breast cancer accounting for 15–20 % of all cases. This subtype of breast cancer is particularly dangerous because there is no specific treatment, it has a less favorable prognosis and a higher grade than other types [4]. TNBC accounts for 5 % of all cancer deaths each year and the median survival

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is 10.2 months. The 5-year survival rate ranges from 65 % for regional tumors to 11 % for metastatic tumors [5]. TNBC is biologically aggressive, with medium/high grade and highly proliferative tumor cells. Compared to other types, it has a higher risk of recurrence, metastasis and mortality. Therefore, early diagnosis is crucial, as it can increase the chances of survival by up to 30 % [6–8]. In clinical practice, screening methods currently used include physical examination of the breast, various types of mammography, ultrasound and magnetic resonance imaging, confirmed by biopsy. However, mammography has limited sensitivity and a high rate of false positives, and repeated use can lead to cumulative radiation exposure. Therefore, the main challenge in TNBC is the development of non-invasive methods for early diagnosis [9–11].

It appears that a key element in the diagnosis and treatment of this group of patients is the identification and analysis of a specific biomarker [12]. However, the quantification of these biomarkers required the use of molecular biology techniques such as enzyme-linked assays, Northern blot, microarrays and quantitative real-time polymerase chain reaction [13,14]. These approaches, although effective, are expensive, time-consuming and require specialized personnel [15,16]. To overcome these challenges, the field of cancer diagnosis has seen a rapid development of technologies that analyze tumors using biomarkers circulating in body fluids, such as blood. This approach, known as liquid biopsy (LB), offers a proactive way for the early detection of cancer, leading to more effective treatments, minimal side effects and improved long-term survival of patients. LB overcomes the limitations of conventional diagnostic methods, enabling ultra-sensitive and selective detection of circulating target molecules in body fluids, such as extracellular vesicles, proteins, nucleic acids and miRNAs [17,18].

Recent technological advances have enabled the identification of miRNAs, a new class of biomarkers that could pave the way for new strategies for early cancer screening. MiRNAs, which play key roles in various biological processes, show altered expression levels in many solid tumors, including breast cancer [19]. These expression levels may be related to chemotherapy resistance. Significant deregulation of miRNA expression is observed not only in tumor and metastatic tissues, but also in the plasma of breast cancer patients, indicating a loss of control of numerous biological processes. Finally, miRNAs may act as specific regulators of metastasis at different stages of the metastatic process, underscoring their importance in the field of oncology [20–22]. In TNBC, specific miRNAs, could serve as potential prognostic biomarkers. miRNA-652 was found to be significantly upregulated in malignant patients compared with benign patients, validating it as a potential biomarker for therapy follow-up [23–25].

In this context, portable (bio)sensors, with a focus on electrochemical ones, have recently received considerable interest due to their many advantages, such as excellent selectivity, cost-effectiveness, non-invasive process and easy interpretation of results [26]. In recent years, various methods for creating electrochemical biosensors for miRNA analysis have been developed. These biosensors are typically realized by immobilizing short capture probes of complementary single-stranded DNA (ssDNA) on the surface of electrochemical transducers. The measurement of hybridization with the target miRNA is done either directly (in the case of label-free electrochemical biosensors) or via changes in the redox signal of an electroactive label [27].

Regarding the detection of miRNA sequences, various electrochemical examples involving different strategies were presented. For instance, in a work by Ebrahimi et al., an electrochemical nanobiosensor was developed for the detection of miRNA-199a in serum. The system consisted of a glassy carbon electrode modified with graphene oxide and gold nanorods, with a thiolated probe attached. The device showed promising results with a linear calibration range from 15 fM to 148pM, a detection limit of 4.5 fM and a standard deviation of 2.9 % [28]. In another work, PNA was immobilized onto a polypirrole layer for recognizing miRNA-21 and silver nanofoam was used to visualize the PNA-miRNA hybridization by performing cyclic voltammetry. This architecture allowed to obtain a detection limit of 0.20 fM with a linear response up to 1 nM [29]. Zouari et al. designed a biosensor using sandwich hybridization of two DNA probes with the target miRNA-21. The biosensor uses two DNA probes and a disposable carbon electrode modified with reduced graphene oxide and gold nanoparticles. The AuNPs, modified with streptavidin and capped with ferrocene, were conjugated with a biotinylated probe containing signal DNA. It has been shown to detect the target miRNA with a detection limit of 5 fM and a linear range between 10 fM and 2pM [30]. In the landscape of miRNAs detection, even if the three examples of biosensors have been characterized with excellent sensitivity and accuracy, it should be highlighted how these methods rely on expensive devices and materials. For instance, glassy carbon electrodes and the three-electrode systems reported, which include a gold working electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode (3 M KCl solution), are known for their outstanding electrochemical properties, but their cost can be prohibitive [31]. In addition, the preparation and modification of these architectures require complex and time-consuming procedures, which may limit their practicality, especially in resource-limited environments or when rapid turnover of experiments is required. Instead, the present work is developed using printed electrodes, which are cheaper and more environmentally friendly than the previous ones [32,33,35,36].

In particular, the present work focused on the development of a paper-based electrochemical device capable of detecting miRNA-652, which is involved in the pathogenesis of TNBCs. The miRNA detection is achieved using AuNPs and an anti-miRNA-652 probe labeled with a redox mediator, namely methylene blue (MB). Nevertheless, in order to improve the practicality and sensitivity of the paper-based biosensor for monitoring miRNA-652, this was coupled with the use of external chromatographic paper-based disk. In particular, Whatman No.1 chromatography paper is used to facilitate the pre-concentration of analytes, due to its porous structure that allows it to retain and concentrate the substances to be examined. With a simple procedure, sensitivity can be improved by using multiple pre-concentration steps involving the drop casting of few microliters of samples on a same disk. As a result, the analytical methodology becomes more sensitive due to the versatility of combining hybrid porous paper-based materials [34]. This platform has demonstrated its effectiveness by successfully detecting miRNA-652 not only in standard solutions but also in human blood serum while maintaining low nanomolar detection limits. This device proves to be particularly useful for the assessment of miRNA-652 levels as a biomarker of TNBC due to several factors: high sensitivity and selectivity, low cost, short procedure time, ease of use, possibility of miniaturization, and wide potential for applicability in complex biological matrices.

2. Experimental section

All the experimental procedures including the reagents, equipment, screen-printed electrodes, modification of the electrode surface and all the experimental setups for carrying out the measurements are reported in the Supporting Information File.

3. Results and discussion

3.1. Optimization of the experimental parameters

The optimization of the experimental parameters for the production of the electrochemical biosensor was performed in a standard phosphate buffered solution (pH 7.4) in order to obtain satisfactory analytical performance towards the target. For each measurement, the percentage change in signal resulting from the interaction between probe and target was evaluated, considering the signal-off architecture of the reported approach [37]. Initially, we focused our efforts on optimizing the amount of AuNPs to be used to change the working electrode area. For this optimization, three volumes were tested: 4, 8 and 12 μL, as shown in

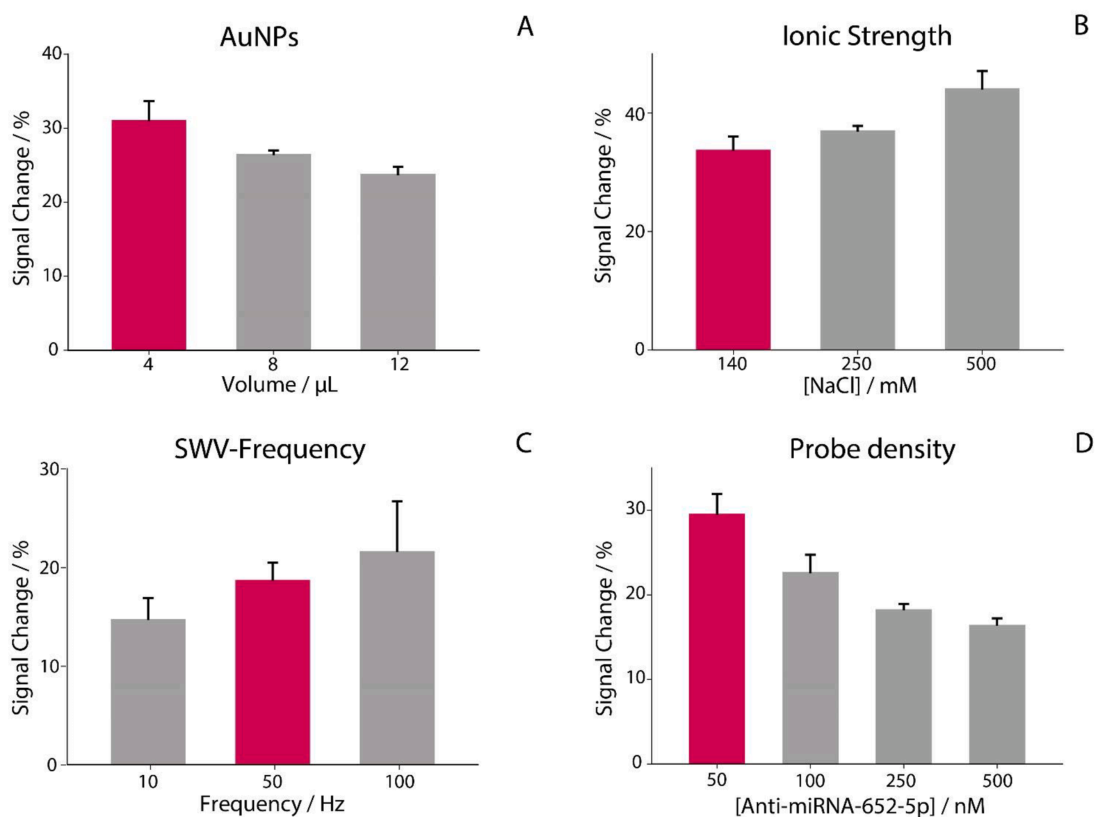

Fig. 1. A) Evaluation of AuNPs volume (4,8, 12 µL), B) Choice of NaCl concentration (140, 250, 500 mM), C) Optimization of the frequency of the square wave (10, 50, 100 Hz), D) Study of anti-miRNA-652 specific probe density (50, 100, 250, 500 nM). All the optimizations have been carried out in the presence of a target concentration of 40 nM.

Fig. 1A.

We decided to continue our development with a volume of 4 µL because with this amount of AuNPs we observed the most significant signal variation, and it can be a consequence of the formation of a thick layer which can limit both the electron transfer of MB and also the anchoring of the DNA probe. Subsequently, the effect of ionic strength was evaluated. Importantly, electrostatic repulsion exists between the target and the probe and it should be considered that both of which are negatively charged [38]. Under such circumstances, a high ionic strength is essential to facilitate effective binding between these two molecules. For this study, we interrogated a solution containing 40 nM of target in presence of 140, 250 and 500 mM of sodium chloride. As shown in Fig. 1B, we selected 140 mM. This choice was guided by the satisfactory sensitivity, e.g. signal variation of ca. 30%. In addition, we found no significant differences from the other concentrations tested, despite the fact 140 mM is already contained in the commercial buffer and no additions are needed. Therefore, we decided to avoid ulterior treatment samples. Next, we refined the frequency of SWV by exploring frequencies comprised between 10, 50 and 100 Hz. As showed in Fig. 1C, a frequency of 50 Hz represented the optimal compromise in terms of signal variation and repeatability. In fact, even if 100 Hz produced a higher mean signal change, this frequency generated noisier voltammograms. Successively, we optimized the probe density, a critically important parameter, as shown in Fig. 1D. As reported in literature, the probe density strongly affects the sensitivity of probe-target recognition [39]. Although the presence of high probe amount produces a very high recorded current, due to the high concentration of MB, the affinity between probe and target is reduced due to the limitation of target diffusion in reaching the probe [40]. Therefore, we decided to continue our study with 50 nM of probe for surface modification. This choice was guided by the satisfactory results were obtained in terms of signal variation due to the higher probe-target affinity, while a lower concentration of probe resulted in a poor electrochemical sensitivity due to the low amount of MB on the electrode.

3.2. Analytical characterization in standard and human serum

After careful evaluation of all key experimental parameters, we systematically examined the analytical performance of the sensing platform. This examination was conducted increasing miRNA-652 target concentrations, ranging from 0.1 to 800 nM, as shown in Fig. 2. Throughout these experiments, we consistently applied the settings we had carefully optimized.

This revealed a characteristic semi-logarithmic sigmoidal correlation between the signal change and the target concentration. Interestingly, both the buffer solution and human serum measurements showed a distinctive sigmoidal trend: this confirmed the effectiveness of our method and its applicability to an untreated biological sample. The correlations were satisfactory, calculated as the R^2, equal to 0.991 and 0.992 for buffer and human serum solutions, respectively. In addition, the limit of detection was calculated as the concentration corresponding to a 10 % signal change and it was approximated to about 0.4 nM. A good repeatability of ca. 5% was obtained (calculated on five replicates in the presence of a target concentration of 50 nM). The results appear promising, with far-reaching impact for the clinical detection of miRNA-652 in terms of ease, production time, sensitivity and cost.

3.3. Selectivity studies

In order to evaluate the selectivity of our platform, we conducted tests in the presence of other three potential interferents, namely miRNA-224 (5′ –uca agu cac uag ugg uuc cgu uuag- 3′), miRNA-21 (5′ –uag cuu auc aga cug agu uga- 3′) and miRNA-29a (5′ –tag cac cat ctg aaa tcg gtt- 3′), as shown in Fig. 3.

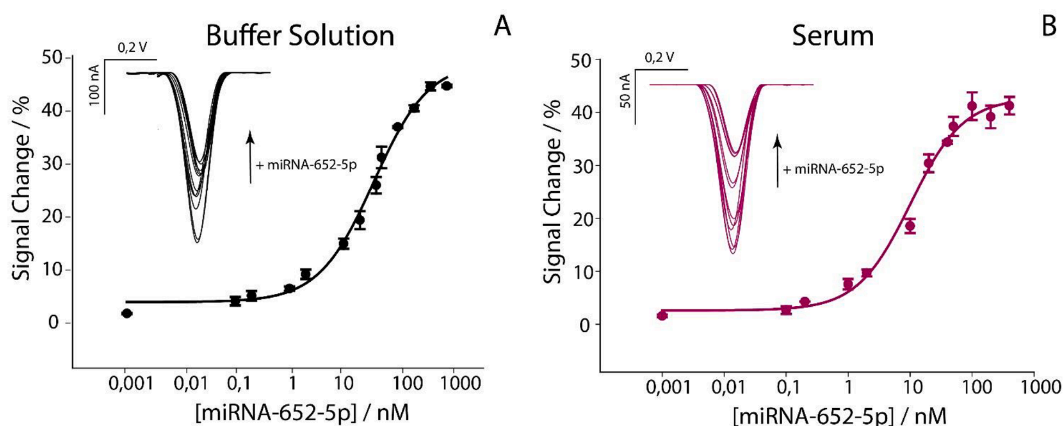

Fig. 2. A) Calibration curve and SWV curves obtained in buffer solution by testing different concentrations of miRNA-652 target from 0.1 to 800 nM. B) Calibration curve and SWV curves obtained in human serum by testing different concentrations of miRNA-652 from 0.1 to 800 nM. All the experiments have been carried out in triplicate. SWV parameters: t eq=5 s, E start = 0.0 V, E end = −0.5 V, E step = 0.001 V, Amplitude = 0.01 V, Frequency = 50.0 Hz.

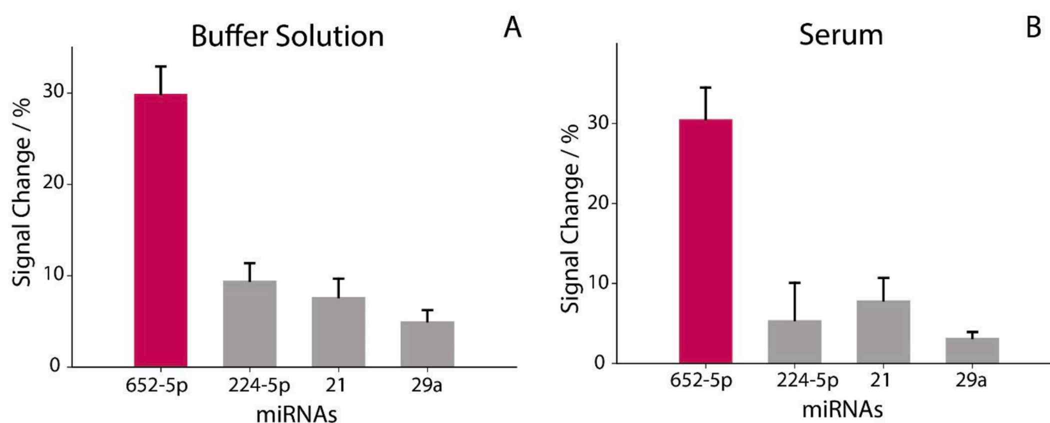

Fig. 3. Selectivity studies comparing signal intensities obtained in the presence of 40 nM miRNA-652 and in the presence of non-complementary RNA strands. A) Buffer solution; B) Human serum.

This evaluation was carried out in both standard solution and untreated human serum, using a constant concentration of 50 nM for each tested species. As can be seen from the histograms, the signal change in presence of the interfering species was not of significant entity if compared with the signal in presence of miRNA-652, thus confirming the high selectivity of the platform.

3.4. *Preconcentration on filter paper discs*

It should be noted that even if the analytical performance are satisfactory, allowing a detection limit of 0.4 nM, the occurring concentration of miRNA in biological fluids is lower: for tumors when some sequences are over-expressed, the concentration can reach also the pM level [41]. Herein, we would like to propose a preliminary evaluation of the possibility to improve the sensitivity without the use of expensive materials/procedure and/or non-sustainable approaches (e.g., extractions, evaporation at high temperature). As reported in this manuscript, the office paper-based platform for miRNA-652 was combined with an external chromatographic paper-based disk, with the goal of pre-concentering the target solution with the use of low-cost strategies. To conduct the study, we examined the sensitivity by adding 10 times a microliter-volume of miRNA-652 target onto a waxed chromatographic paper-based disk characterized with a diameter of 3 mm. Each of the following drop was applied after the solvent from the previous drop evaporated. To demonstrate the applicability of this method, we performed pre-concentration studies using two target concentrations, 0.1 and 1 nM, in both standard and human serum solutions, as reported in Fig. 4.

After the target was pre-concentrated, the content was released into the working solution that will be subsequently placed onto the SPE and analyzed. The results obtained showed the effectiveness of the pre-concentration process in improving the detection limits of the sensing platform of ca.10-fold. In fact, the concentration of 0.1 nM that was below the calculated detection limit produced a signal change variation comparable to the one obtained in presence of 1 nM (as reported in Fig. 4A and C). The same happened while analyzing a 10x pre-concentrated 1 nM target solution, which appeared as the signal of a 10 nM (Fig. 4B and D).

4. Conclusions

In this work, for the first time, an electrochemical paper-based screen-printed electrode has been designed, characterized and applied towards the detection a promising biomarker involved in the pathogenesis of TNBC, namely miRNA-652. The three-electrode system has been screen-printed onto office paper, and it has been modified with AuNPs and an anti-miRNA probe for the selective recognition of the chosen target. After having optimized the most relevant experimental parameters, the electrochemical biosensor was able to detect miRNA-652 down to ca. 0.4 nM in both standard and human serum solutions. The repeatability and the selectivity of the platform were satisfactory, and the system has been improved in its sensitivity with the use of an

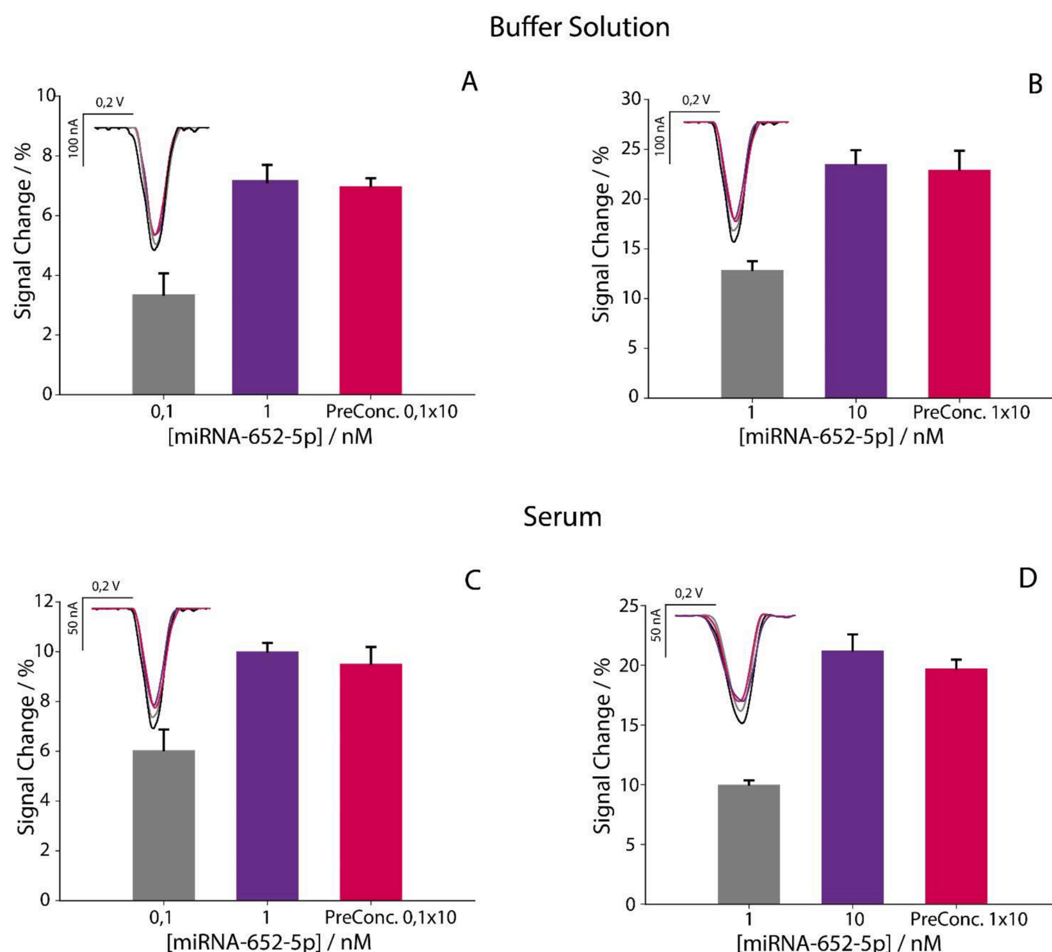

Fig. 4. Preconcentration of miRNA-652 0,1 nM in A) phosphate buffer solution and C) human serum; Preconcentration of miRNA-652 1 nM in B) phosphate buffer solution and D) human serum.

innovative/frugal external chromatographic paper-based disk. It should be noted that, by just using external wax-patterned Whatman No.1 disks to pre-concentrate sample, the sensitivity towards miRNA detection resulted amplified of 10-fold; this preliminary approach was satisfactorily applied to both standard and human serum solutions, improving the LOD of 10-fold with the adoption of only 10 steps of pre-concentration. The entire system, which integrates an office paper-based screen-printed electrode for miRNA detection and an external chromatographic paper-based disk for pre-concentration, was conceived as a disposable device capable of providing immediate feedback in the field of liquid biopsy, with the potentiality of shortening the time between diagnosis and treatment of such a devastating disease as triple-negative breast cancer. In particular, the adoption of the pre-concentration steps could be used in a programmable way to obtain the desired sensitivity.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.electacta.2024.144205.

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